

Defining the Functions of CASK in Skeletal Muscle

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Abstract

Traditionally, the mammalian neuromuscular junction, the primary synapse found in skeletal muscle, has been termed the “simple synapse” for its basic chemical signaling mechanisms as compared to central nervous system synapses. However, recent data has suggested that the neuromuscular junction is far more complex than initially perceived and that various proteins are involved in the orchestration of synaptic function and development. One such protein, Ca^{2+} /calmodulin associated serine/threonine kinase (CASK), has been previously identified as a key component in neuronal development. Our lab has found that CASK is concentrated at the neuromuscular junction, cytoplasm, and nuclei of skeletal muscle fibers, where evidence suggests that it may play a role in the structural scaffolding of synapses, receptor trafficking, protein phosphorylation, and transcription. To further elucidate the mechanisms of CASK in skeletal muscle, we have generated two distinct conditional knockout murine models of CASK in skeletal muscle. One model represents a knockout of non-synaptic CASK, while the other model represents a knockout of CASK in both the cytoplasm and at the neuromuscular junction. Recent data has shown that mice without CASK at the neuromuscular are not born at the predicted Mendelian ratios, while mice only deficient of cytoplasmic CASK show no observable phenotype. Further analyses of these models will be necessary to help to define the function of CASK in skeletal muscle. Studying the neuromuscular junction is the key to understanding the underlying mechanisms of neuromuscular diseases. Delineating the functions of CASK in skeletal muscle may lead to the identification of novel treatment approaches for neuromuscular diseases.

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I

Introduction

Significance

Neuromuscular disorders encompass a large number of diseases with a wide array of etiology and severity, many of which are fatal in childhood. With the exception of few, most of these diseases are incurable, and will result in eventual death due to gradual muscle weakness, breathing problems, and/or heart failure. Neuromuscular diseases are caused by abnormalities in skeletal muscle, motor neurons, or the neuromuscular junction; yet many of the underlying molecular mechanisms of these diseases have yet to be determined (Bushby et. al. 2009, Lee et. al. 2009, McConville and Vincent 2002, Sandona et. al. 2009, Stavarachi et. al. 2010, Turner et. al. 2010). Currently, there are few effective treatments for neuromuscular diseases (Aartsma-Rus and van Ommen 2010, Muir and Chamberlain 2009, Tang et. al. 2010, Trollet et. al. 2009, Wood et. al. 2010).

The key to understanding and learning more about the mechanisms behind such diseases lies at the interface between the nervous system and skeletal muscle: the mammalian neuromuscular junction. Since nearly every neuromuscular disorder has an underlying genetic basis, an in depth understanding of protein function at the neuromuscular junction is of utmost importance when defining the molecular mechanisms of disease (Cardamone et. al. 2008). Many proteins have been characterized at the neuronal synapse, helping to elucidate the mechanisms behind several neurological diseases. Additional knowledge of the functions of newly identified classes of proteins at the neuromuscular junction that are involved in skeletal muscle and neuromuscular junction development may lead to the identification of novel treatment approaches for neuromuscular diseases.

Muscle Development

The embryonic development of skeletal muscle requires a coordinated regulation of various cell types and myogenic factors. In the developing embryo, cell clusters called somites arise from the mesoderm to eventually differentiate into three cell types: skin, vertebrae, and skeletal muscle. Cells located in the dorsal part of the somites begin to form the dermomyotome, which is responsible for forming the musculature. The dermomyotome will divide to form myoblasts, or muscle progenitor cells, which through the action and up- or down-regulation of several myogenic regulatory factors, notably Myf5 and MyoD, will fuse together to form myofibers, the cellular units of mature skeletal muscle (Figure 1-1) (Bakkar and Guttridge 2010, Buas and Kadesch 2010, Gros et. al. 2005, Hollway and Currie 2005, Krauss et. al. 2005, Otto and Patel 2010, Te and Reggiani 2002).

Adult skeletal muscle is made up of many bundles of myofibers. Myofibers are multinucleated, cylindrical structures with nuclei that characteristically localize to the periphery of the cell membrane. Myofibers can further be broken down into repeating units called sarcomeres, which are organized arrangements of the proteins actin and myosin (Bloch et. al. 2002, Kee et. al. 2002, Luther 2009, Pinotsis et. al. 2009, Sanger and Sanger 2008, Sparrow and Schock 2009). These proteins are responsible for the contractile properties of skeletal muscle, and function in response to membrane depolarization and a corresponding increase in intracellular calcium levels (Endo 2009, Hezog 2008, Rosenberg 2009).

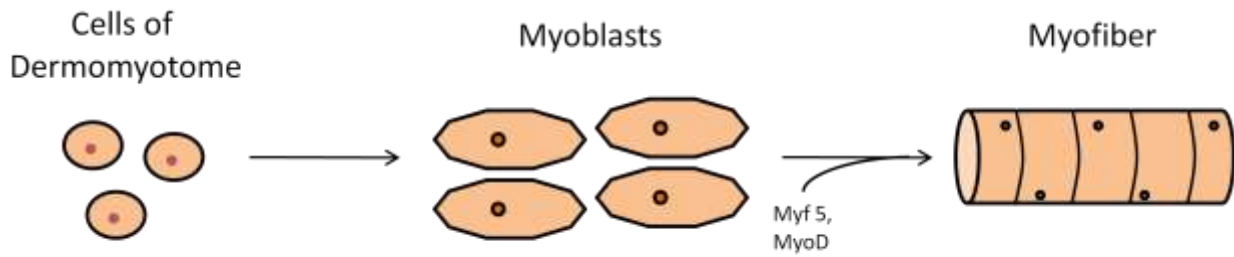


Figure 1-1: During development, mesodermal cells of the dermomyotome will divide to form the muscle progenitor cells, myoblasts. Upon chemical signaling from a number of myogenic regulatory factors, myoblasts will differentiate and fuse together to form mature myofibers, the basic cellular units of skeletal muscle. Myofibers are multinucleated with nuclei localizing to the periphery of the cell membrane.

Synaptic Scaffolding and the Neuromuscular Junction

The mammalian neuromuscular junction (NMJ) refers to the site where a skeletal muscle fiber is innervated by a single motor neuron. When the brain initiates the response to move, a nerve impulse is conducted through the central and peripheral nervous systems to the NMJ. At this location, the presynaptic motor neuronal axon releases acetylcholine (ACh), a major excitatory neurotransmitter in the body responsible for membrane depolarization. ACh travels across the synapse where it binds to homogenous receptors embedded within the invaginated postsynaptic membrane of a skeletal muscle fiber. ACh receptors are located at the tops of the folds of the invaginated skeletal muscle, referred to as the primary gutter. Binding of ACh to these receptors produces a rise in intracellular calcium levels and subsequent muscle contraction (Burden 1998, Hughes 2006, Glass et. al. 1996, Glass et. al. 1997, Madhavan and Peng 2003, Madhavan 2005, Meyer and Wallace 1998, Patton 2003, Ruegg and Bixby 1998, Sanes and Lichtman 1999, Wu 2010).

ACh receptor complexes play a crucial role in synaptic scaffolding and function of the vertebrate NMJ. However, there are various other proteins that are involved in the scaffolding, stabilization, development, function, and orchestration of this chemical synapse. One such family of proteins found in cell-cell junctions, such as the NMJ, are the membrane-associated guanylate kinases (MAGUKs), which share the defining characteristic of containing multiple protein-protein interaction domains. MAGUKs are believed to interact with each other, as well as channels and receptors, via their PDZ domains, which were coined after the proteins in which they were first discovered (PSD-65, Discs Large, and Zo-1). In addition to PDZ, MAGUKs also contain a number of other protein-interactor domains, including a HOOK domain, an SH3 domain (Src homology 3), a guanylate kinase domain (GK), and two L27 domains (initially discovered in the proteins lin-2 and lin-7) (Anderson 1996, Craven et. al. 1998, Garner et. al. 2000, Kim et. al. 2004, Kornau et. al. 1997, Pawson et. al. 1997). One notable MAGUK protein, Ca²⁺/calmodulin-associated serine/threonine kinase (CASK), has been proven to facilitate the development of cell junctions throughout the body and is essential to sustain life (Lavery and Wilson 1998). Recently, CASK has been shown to be present throughout skeletal muscle fibers and concentrated at the NMJ, where we hypothesize that it plays a major role in synaptic development and/or function (Sanford et. al. 2004, Sanford et. al. 2008).

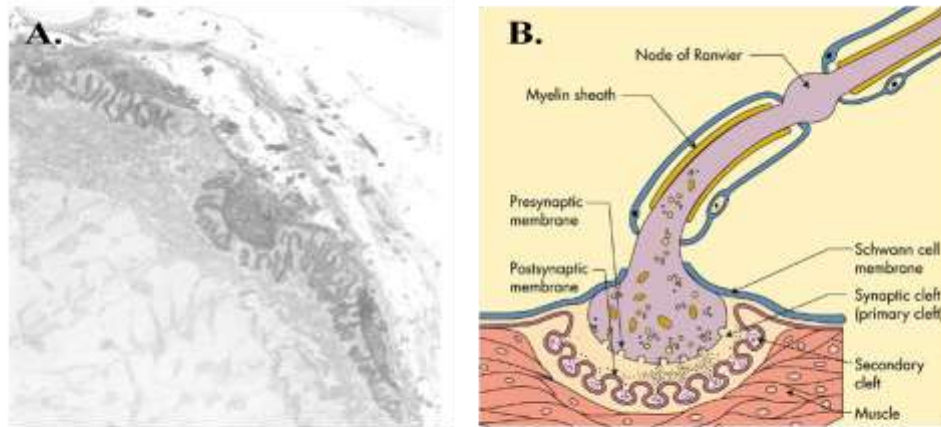


Figure 1-2: Two distinct depictions of the mammalian neuromuscular junction. A.) An electron micrograph of the NMJ. B.) A schematic representation of the NMJ, courtesy of www.images.md

An Overview of CASK

CASK is composed of every characteristic MAGUK domain, and is unique in that it also contains an N-terminal CaMK (Ca^{2+} /calmodulin kinase-like) domain. The PDZ domain of CASK is one of the main indicators of its involvement in synaptic scaffolding (Daniels et. al. 1998). CASK has been shown to bind and interact with neurexin-1, a postsynaptic scaffolding protein that aids in the structural development of the synapse via its binding to the presynaptic protein neuroligin (Biederer et. al. 2001, Hata et. al. 1996, Zhang et. al. 2001). CASK also has the ability to cluster inward-rectifying potassium channels and form complexes with other structural proteins by means of its PDZ domain (Kim et. al. 1995, Leonoudakis et. al. 2001, 2004). Its other characteristic MAGUK domains are also believed to be involved in the interaction and trafficking of other various proteins (Biederer et. al. 2001, Borg et. al. 1998, Daniels et. al. 1998, Dimitratos et. al. 1997, Ferguson et. al. 1985, Hata et. al. 1996, Hoskins et. al. 1996, Maximov et. al. 1999, Leonoudakis et. al. 2004, Zhang et. al. 2001). The HOOK domain, notably, is thought to confer an intermolecular binding between the SH3 and GK domain of CASK, a

potential means by which the CASK protein may biochemically regulate its own trafficking, localization, and protein interactions (Hoover et. al. 2000, Kohu et. al. 2002, McGee et. al. 1999, 2001, Tavares et. al. 2001).

CASK is an X-linked protein that was first discovered in mammals based on its interaction with neurexin, thought to function in stabilizing the neuronal synapse (Biederer et. al. 2001, Hata et. al. 1996, Sun et. al. 2009, Zhang et. al. 2001). It has also been previously identified as a key protein in the development of *C. elegans*. Lin-2, the CASK homolog in *C. elegans*, demonstrates the first *in vivo* evidence of the capability of CASK to cluster potassium and calcium channels by means of its PDZ domain. It was in this model that CASK was shown to form a tripartite complex with two other PDZ domain-containing proteins, Mint and Veli (Borg et. al. 1998, Butz et. al. 1998, Kaeck et. al. 1998, Sinske et. al. 1996). This complex has been proposed to serve as a mechanism for the recruitment of calcium channels and other receptors, as well as a means to facilitate vesicle exocytosis (Butz et. al. 1998, Maximov et. al. 1999, Schuh et. al. 2003).

The GK and CaMK domains of CASK share a similar structural and sequence homology with traditional kinases, however, until recently CASK was not thought to be involved in phosphorylation. The presence of the CaMK domain had led to the hypothesis that CASK may be involved in the phosphorylation of the neuron-specific adhesion molecule, neurexin, as well as certain other protein interactors, such as Discs large (Dlg). CASK's inability to bind Mg^{2+} , a traditional catalyst for phosphorylation, had previously led to the speculation that CASK may be a pseudokinase with phosphorylative abilities yet to be determined. However, recent evidence suggests a potential kinase activity of CASK despite its lack of Mg^{2+} -binding sites (Mukherjee et. al. 2008, 2010).

CASK's unique combination of domains allow for it to have a complex and adaptive role within the changing environment of the developing skeletal muscle. The essential role of CASK in development has been demonstrated through models in which a complete knockout of CASK protein in mouse results in cleft palate and neonatal death (Lavery and Wilson 1998). More recently, disruptions in the CASK protein have also been linked to a variety of X-linked mental retardations, further illustrating its important function in development (Bailey and Aldinger 2009, Hackett et. al. 2010, Hayashi et. al. 2008, Hsueh 2009, Najm et. al. 2008, Piluso et. al. 2009, Tarpey et. al. 2009). More pertinent to muscle development, our lab has shown that CASK localizes to the nucleus in developing myoblasts, while migrating to the cytoplasm in differentiated myotubes, suggesting a mechanism of protein trafficking during development (Gardner et. al. 2006). The potential ability of the CaMK domain to function as a kinase without functional Mg^{2+} binding sites may provide a means for the phosphorylation of other proteins when traditional kinases exhibit a depressed function in the Mg^{2+} -poor environment of developing skeletal muscle.



Figure 1-3: The functional domains of CASK. CASK consists of the traditional MAGUK domains: a PDZ binding domain, an Src homology 3 (SH3) domain, a HOOK domain, a guanylate kinase (GK) domain, and two L27 domains. Unique to CASK is the Ca^{2+} /calmodulin kinase-like (CaMK) domain, which may be involved in phosphorylation.

CASK has already been shown to function as a scaffolding protein in central nervous synapses of the brain, and is capable of functioning as a kinase and transcription factor (Huang et. al. 2009, Hsueh et. al. 2000, Wang et. al. 2004a, 2004b). The presence of CASK in skeletal muscle and at the neuromuscular junction suggests that it may play a similar role at this location. Its combination of functional protein-interaction domains suggests it may play an adaptive role in muscle development, and interaction with various other proteins may allow for CASK to play an important role in formation and stabiliztion of the NMJ, as well as receptor trafficking and cell function.

Objectives

The goal of this study is to focus on defining the functions of the MAGUK protein CASK in skeletal muscle and at the neuromuscular junction. The overarching hypothesis that CASK is involved in the structural scaffolding of synapses, receptor trafficking, and protein phosphorylation throughout developing skeletal muscle will be tested in two distinct conditional knockout models: one that exhibits a loss of cytoplasmic CASK and the other that exhibits a complete loss of CASK in skeletal muscle in the cytoplasm and at the neuromuscular junction. Specifically, the study seeks to characterize a phenotype associated with each model, determine CASK's ability to alter the levels of various other protein interactors, and CASK's ability to influence the phosphorylation of other known NMJ proteins. This comprehensive characterization of CASK's function at the NMJ will help to elucidate the molecular mechanisms by which abnormalities in NMJ and skeletal muscle development can lead to neuromuscular disease.

II

Characterization of Non-Synaptic CASK Function in Skeletal Muscle

Introduction

In adult murine skeletal muscle, CASK protein is found localized to the membrane, cytoplasm, select nuclei, and is most enriched to the post-synaptic side of the NMJ (Sanford *et. al.* 2004). However, many of its functions in skeletal muscle have yet to be defined. CASK, via its PDZ domain, has already been shown to play a role in receptor trafficking to the synapse in neuronal cultures (Hata *et. al.* 1996, Leonoudakis *et. al.* 2001, 2004). CASK adopts a conformation that can bind calcium channels and NMDA receptor subunits, a subtype of glutamate receptors, and localizes them in clusters to neuronal synapses in culture (Maximov *et. al.* 1999, Nagano *et. al.* 1998). CASK is also thought to stabilize the neuronal synapse via its interaction with neurexin-1 (Hata *et. al.* 1996). The presence of CASK in the cytoplasm of skeletal muscle and at the post-synaptic membrane leads us to formulate the hypothesis that CASK may also be involved in channel clustering and protein trafficking to the mammalian NMJ (Sanford *et. al.* 2004, Sanford *et. al.* 2008).

Additionally, it has been shown that CASK interacts *in vivo* in skeletal muscle with another MAGUK protein, Discs large (Dlg) (Sanford *et. al.* 2004). Dlg has also been shown to bind glutamate receptors and potassium channels, and its formation of a complex with CASK at the NMJ further supports the role that CASK may play in protein trafficking in skeletal muscle (Nagano *et. al.* 1998, Tejedor *et. al.* 1997). Dlg also contains a PDZ domain, and has also been implicated in the trafficking of receptors to synapses (Lue *et. al.* 1996). Evidence suggests that CASK and Dlg may share a similar role *in vivo*, and knockouts of either CASK or Dlg protein function in mice show similar neonatal lethal phenotypes (Lee *et. al.* 2002, Nix *et. al.* 2000).

CASK and Dlg also form a tripartite complex with the inward-rectifying potassium channel subunit Kir2.2, also suggesting their ability to cluster channels (Leonoudakis *et. al.* 2001, 2004). We hypothesize that CASK interacts with other MAGUK proteins, protein channels, and receptors in skeletal muscle, and we set out to delineate the functions of CASK *in vivo*.

One widely used method of protein characterization is the generation and study of a total knockout (KO) mouse model, in which the gene is disrupted and the encoded protein is absent in all tissues. A resulting phenotype then can be characterized to determine the function of the protein of interest *in vivo*. CASK was first knocked-out by Lavery *et al* due to a transgenic insertion in the CASK locus. These CASK KO mice exhibited cleft palate and died within 24 hours of birth (Lavery *et. al.* 1998). However, muscle tissue was not studied in this initial CASK KO model, and the phenotype was not associated with CASK function in a specific tissue.

Another widely used method to delineate protein function *in vivo* is through the generation of conditional knockout (cKO) murine models. In this instance, the gene can be disrupted only in a specific tissue, so that the resulting phenotype can be attributed to the loss of function of the target protein in a specific tissue. To determine the role that CASK plays in skeletal muscle, our lab generated cKO models of CASK in which the protein was ablated specifically in skeletal muscle tissue. This was done so that we could attribute any resultant phenotype to skeletal muscle pathology. To develop these models, we used a method known as the Cre/lox recombination system (Sternberg *et. al.* 1981). This system requires the insertion of two palindromic genetic sequences known as “locus of crossover P1” (*loxP*) sites on the same chromosome and the presence of an enzyme known as *Cre* recombinase (*Cre*). When the *loxP* sites are inserted around a portion of a gene, (at which point the gene is termed “floxed”), *Cre* will cause a recombination event between the two *loxP* sites, excising the intervening DNA and

leaving one *loxP* site remaining, effectively interrupting the gene (Hoess *et. al.* 1985). The floxed gene will be present in every cell in an organism; however, the *Cre* transgene can be linked to a specific promoter activated only in certain tissues to ensure tissue-specific protein ablation. To achieve skeletal muscle specific CASK gene knockout, we obtained a mutant line of mice that exhibited a floxed portion of the CASK gene that was initially developed by Atasoy *et al.* We then mated these mice with mice expressing *Cre* recombinase from the Human Skeletal Actin (HSA) promoter, a promoter expressed in terminally differentiated skeletal muscle fibers (Miniou *et. al.* 1999). In the resulting experimental mice (CASK SkM cKO), *Cre* will only be expressed in skeletal muscle to generate a skeletal muscle specific ablation of CASK. Study of these mice will allow us to test hypotheses about CASK functions in skeletal muscle.

Due to the multiple proposed physiological roles of CASK, including receptor trafficking, protein phosphorylation, transcription, and synaptic scaffolding, we postulate that a complete ablation of CASK protein in skeletal muscle will result in a degenerative and pathological phenotype (Hata *et. al.* 1996, Leonoudakis *et. al.* 2004, Mukherjee *et. al.* 2008, Sanford *et. al.* 2004). We hypothesize that CASK plays an essential role in skeletal muscle by binding to and trafficking receptors and protein channels to the NMJ.

Methods

CASK Knockout Mice and the HSA Transgenic Line

Mice possessing a floxed allele with *lox P* sites surrounding exon 1 of the CASK gene on the X-chromosome were generated by Atasoy *et al.* and made available for purchase from Jackson Laboratories. These mice were used to breed the CASK SkM cKO mouse model. The HSA-Cre transgenic mice were generated by Miniou *et al.* and possess the Cre recombinase transgene linked to the muscle-specific promoter human skeletal actin (HSA). In order to generate the CASK SkM cKO mice, we first bred female CASK flox mice to male mice carrying the HSA-Cre transgene (Cre⁺), producing an F1 generation that resulted in both wild-type (WT) and CASK flox mice (flox) with or without the HSA-Cre transgene. These genotypes are referred to as follows: Male mice possessing a floxed CASK allele and the HSA-Cre transgene are CASK cKO males (cKO), CASK flox males without the Cre transgene (flox/-), females heterozygous for the floxed allele with the Cre transgene (Het/+), or without (Het/-), and wild type mice with the Cre transgene (WT/+), or without (WT/-). Because CASK is an X-linked gene, the breeding scheme favors male knockouts. Experiments were conducted on CASK cKO males with WT/+, WT/-, and flox/- littermate controls. Subsequent matings were maintained by mating Het/- females to WT/+ males.

All mice are housed in a fully-equipped vivarium at The Ohio State University under an IACUC approved protocol to Dr. Jill Rafael-Fortney. Mice are kept on a standard 12 hr day/night cycle and were provided food (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories) and water *ad libitum*.

Genotyping of Mice

Genomic DNA was obtained from tail biopsies of mice and analyzed via the polymerase chain reaction (PCR). To test for the presence of the HSA-Cre transgene, primers were designed to screen for a DNA sequence within the *Cre* region, as initially described by Miniou *et al.* This first pair of primers are as follows: HSA-Cre Forward (5' CCGGTCGATGCAACGAGTGAT 3') and HSA-Cre Reverse (5' ACCAGAGTCATCCTTAGCGCC 3'), resulting in a 790 bp product at a 56°C annealing temperature. Genotyping for presence of the floxed allele was performed using two sets of primers. The first primer set was designed to screen for a region within the neomycin resistance (*Neo*) cassette, and are as follows: CASK Neo Rejuv Forward (5' GCTATGACTGGGCACAAC 3') and CASK Neo Rejuv Reverse (5' GCCAACGCTATGTCCTG 3'), yielding a 610 bp product at 56°C annealing temperature. A second primer set was used in conjunction with CASK cKO Rejuv F/R to confirm the presence of the floxed allele, and are as follows: IMR 6389 (5' CGCATCGCCTTCTATCGCCTTCTT 3') located within the *Neo* cassette and IMR 6390 (5' CACCCACCCACTCATGCTTCTGC 3') located within the CASK genomic sequence, yielding a 256 bp product at 58°C annealing temperature. To test for the presence of the wild type allele, primers were designed to screen for a DNA sequence around the Neo cassette of the targeting sequence generated by the Jackson Labs. Primers included in this set are as follows: SSC 221 (5' AGGCCTTGCAAATCATCGTTCAT 3') and IMR 6388 (5' CTCTCCAAATCTCCTTACCTCAA 3'), yielding a 490 bp product for the WT allele and a 1,400 bp product for the floxed allele at 60°C annealing temperature. All PCR reaction products were run on 1% agarose gels (0.02% ethidium bromide) and photographed under ultraviolet light.

Histological Analysis

Unfixed quadriceps muscles were dissected from WT, Flox, and CASK cKO mice at 12 weeks of age and embedded in OCT (optimal cutting temperature) cryoprotective mounting medium (Tissue-Tek, Hatfield, PA). Tissues were then slow-frozen on liquid nitrogen-cooled isopentane and stored at -80°C. Frozen tissue was cut into 8 µm cross-sections on a cryostat. To view the histology of the section, slides were fixed in 100% ethanol and stained with hematoxylin and eosin (H&E) using a standardized protocol.

Immunohistochemistry

Immunohistochemical analysis was conducted on 8 µm cross-sections of unfixed frozen quadriceps from WT, Flox, and CASK cKO mice aged 12 weeks. Sections were first blocked in 1% gelatin in potassium-phosphate buffered saline (KPBS) for 15 min before being washed in KPBS for 15 min. Following the washes, sections were incubated for 2 hours in a primary antibody solution consisting of primary antibody diluted in KPBSG (KPBS + 0.5% gelatin) plus normal goat serum (1%). Primary antibodies used include: CASK polyclonal (Zymed, 1:25), Dlg polyclonal (a kind gift from A. Chishti, 1:1,250), and Grip polyclonal (Chemicon, 1:50). Sections were then washed 3 x 5 min in KPBSG. Following rinsing, sections were incubated in a secondary antibody solution that consists of CY3 anti-rabbit secondary antibody (Jackson Laboratories, 1:200), Alexa 488-conjugated α -bungarotoxin (Molecular Probes, Inc., 1:1000), normal goat serum (1%), in KPBSG for 1 hour. α -bungarotoxin was used to fluorescently label the NMJ. After incubation, sections were once again washed in KPBSG 3 x 5 min. Slides were then mounted in Vectashield® (Vector Labs, Burlingame, CA) and 4',6-diamidino-2-phenylindole (DAPI, 500 ng/mL), a DNA-tagging fluorescent dye used for nuclei visualization,

before being coverslipped. Images were captured with a Nikon Eclipse E800 microscope and a SPOT-RT slider digital camera and SPOT® software.

To view the topology of the neuromuscular junction, quadriceps from 12 week old mice were dissected and preserved in 1% paraformaldehyde in KPBS for 1 hour, followed by immersion in 20% sucrose in KPBS overnight. Tissue was then embedded in mounting medium and frozen down on liquid nitrogen-cooled isopentane, as done previously. 30 μ m transverse sections of quadriceps were cut and placed onto slides. For immunohistochemical analysis, slides were equilibrated in KPBS for 5 min and washed in 0.1 M glycine in KPBS for 1 hour. Sections were then extracted on ice in 0.5% Triton X-100 in KPBS for 5 min. Slides were then washed in KPBS and blocked in 1% gelatin in KPBS for 1 hour. To fluorescently label the NMJ, sections were incubated for 3 hours in an antibody solution consisting of Alexa-488 conjugated α -bungarotoxin (1:200) and normal goat serum (1%) in KPBSG. Following incubation, sections were washed 3 x 15 min in KPBS with 0.1% Tween-20, mounted in Vectashield, and coverslipped. The circumference of each NMJ in every section was traced and quantitatively measured using SPOT® software. Sizes were averaged and compared across the different genotypes.

Confocal analysis was performed by Jamie Sanford on 8 μ m cross-sectioned quadriceps from 12 week old mice that had been fixed in 1% paraformaldehyde and 20% sucrose and frozen, as described above. Slides were then equilibrated in KPBS, washed in 0.1 M glycine, and extracted in 0.5% Triton X-100, as done when staining for NMJ topology. Slides were blocked in 1% gelatin in KPBS for 1 hour and incubated in primary antibody solution, consisting of primary antibody diluted in KPBSG plus 1% normal goat serum. Primary antibodies include CASK polyclonal (Zymed, 1:25) and Dlg polyclonal (a kind gift from A. Chisti, 1:1,250). Slides

were washed 3 x 15 min in KPBS and incubated in a secondary antibody solution that consists of CY3 anti-rabbit secondary antibody (Jackson Laboratories, 1:200), Alexa 488-conjugated α -bungarotoxin (Molecular Probes, Inc., 1:1000), and normal goat serum (1%) in KPBSG for 3 hours. Slides were washed in 0.1% Tween-20 in KPBS 3 x 15 min and mounted in Vectashield and coverslipped.

Subcellular Fractionation

Dissected skeletal muscle tissue was homogenized in 7.5 volumes of Homogenization buffer (20 mM sodium pyrophosphate, 20 mM sodium phosphate monobasic, 1 mM MgCl_2 , 303 mM sucrose, 0.5 mM EDTA, pH 7.1) and then centrifuged for 15 min at 14,000 x g. The supernatant was then collected and filtered through cheesecloth to isolate the S1 fraction, which consists of cytoplasmic organelles. This fraction was then centrifuged for 37 min at 142,413 x g and the supernatant was discarded. The pellet was then resuspended in 70% original volume of Resuspension Buffer I (0.6 M KCl, 0.3 M sucrose, 50 mM Tris-Cl, pH 7.4, 0.1 mM PMSF protease inhibitor, 0.75 mM benzamidine) and centrifuged again for 37 min at 142,413 x g. Following the spin, the supernatant was collected and saved as the S2 fraction, also consisting of cytoplasmic organelles, and the pellet was resuspended in 0.3 volumes of Resuspension Buffer II (10 mM sodium phosphate, 5 mM EDTA, 150 mM NaCl, pH 7.8). The resuspended was saved as the isolated microsome fraction, consisting of membrane-bound proteins. Western analysis could then be performed on homogenates, S1, S2, and microsome fractions to determine the levels of protein in each different cell fraction.

Nuclear extract (NE) fractions were also isolated separately from total skeletal muscle homogenates. Homogenates were resuspended in CE buffer (10 mM HEPES, pH 7.6, 60 mM KCl,

1 mM EDTA, protease inhibitors) plus NP-40 nonionic detergent (0.25%) and incubated for 5 min on ice. Samples were then spun at 718 x g for 4 min at 4°C. The pellet is then isolated and washed in CE buffer without NP-40 and spun for another 4 min at 718 x g at 4°C. The pellet was then resuspended again in CE buffer without NP-40 and carefully layered on top of 2 mls of Buffer B (30% sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 7.5, 2 mM EDTA, 0.15 mM spermine, 0.75 mM spermidine, 1 mM DTT). Samples were then spun at 1,643 x g for 15 min at 4°C. The pellet was isolated and resuspended in CE buffer without NP-40 and carefully layered on top of 2 mls of Buffer C (same as Buffer B with 50% sucrose). Samples were again spun at 1,643 x g for 15 min at 4°C before the pellet was isolated and resuspended in 200-300 µL of NE buffer (20 mM Tris pH 8.0, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, protease inhibitors). Samples were then incubated on ice for 10 min before spun down once more a 1,643 x g for 10 min to pellet anything insoluble. The supernatant was then saved as the nuclear extract (NE) fraction and stored at -80°C.

Western Analysis

Total skeletal muscle was collected from 12-week old WT, Flox, and CASK SkM cKO mice and homogenized in Newcastle buffer (75 mM Tris, pH 6.8, 3.8% sodium dodecyl sulfate (SDS), 4 M urea, 20% glycerol). Protein concentrations were determined using the DC protein assay (Biorad) followed by subsequent absorbance measurements at 750 nm wavelength. 50 µg of protein per sample was then run out on 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels at 80 V for approximately 3 hours. After separation, proteins were electrically transferred from SDS-PAGE gels to nitrocellulose (Whatman International) at 80 V for 90 min using a wet transfer apparatus (Bio-Rad). Nitrocellulose Western blots were then blocked in 3%

bovine serum albumin (BSA) in Tris-buffered saline plus 0.1% Tween-20 (TBST) and 1% normal goat serum for 1 hour. Blots were then incubated in a primary antibody solution consisting of a primary antibody and 1% normal goat serum in TBST for 2 hours. Primary antibodies include CASK polyclonal (Zymed, 1:250), Dlg monoclonal (Transduction Laboratories, 1:250), and Pan-Maguk monoclonal (UC Davis, 1:250). Blots were then washed 3 x 15 min in TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Jackson Laboratories, 1:10,000) in TBST with 1% normal goat serum for 1 hour. Chemiluminescence (Amersham) was used for detection of bound primary antibody.

Physiological Analysis

The following procedure was performed by the laboratory of Dr. Paul Janssen. Extensor digitorum longus (EDL) muscle was dissected, tendon to tendon, from 12-week old WT/+ and CASK SkM cKO mice. Muscles were attached horizontally by their tendons to a force transducer and an electromagnetic length adaptor in Krebs solution. Optimum length of the EDL was determined by subjecting the muscle to successive twitches and measuring the length at which maximum isometric force is achieved. Maximum tonic contraction was measured by subjecting the muscle to 5 tetanic stimulations of 350 ms at 2 minute intervals, using a stimulation frequency of 125 Hz. Measurements were expressed per unit cross-sectional area (mN/mm^2).

Results

Generation of HSA-Cre CASK SkM cKO Mice

The CASK SkM cKO mice were generated via the Cre/lox recombination system. Mice that possess the floxed CASK allele were mated to mice that express the *Cre* transgene from the HSA promoter to interrupt the CASK gene specifically in skeletal muscle tissue. This generates a generation of mice that include: CASK SkM cKO males, flox/- males, het/+ females, het/- females, and WT/+ and WT/- mice (figure 2-2). Genotypes of mice were observed at the expected Mendelian ratios. CASK SkM cKO mice showed no obvious muscle weakness, labored breathing, or gross phenotypes. Mice of each genotype were viable for at least 12 months.

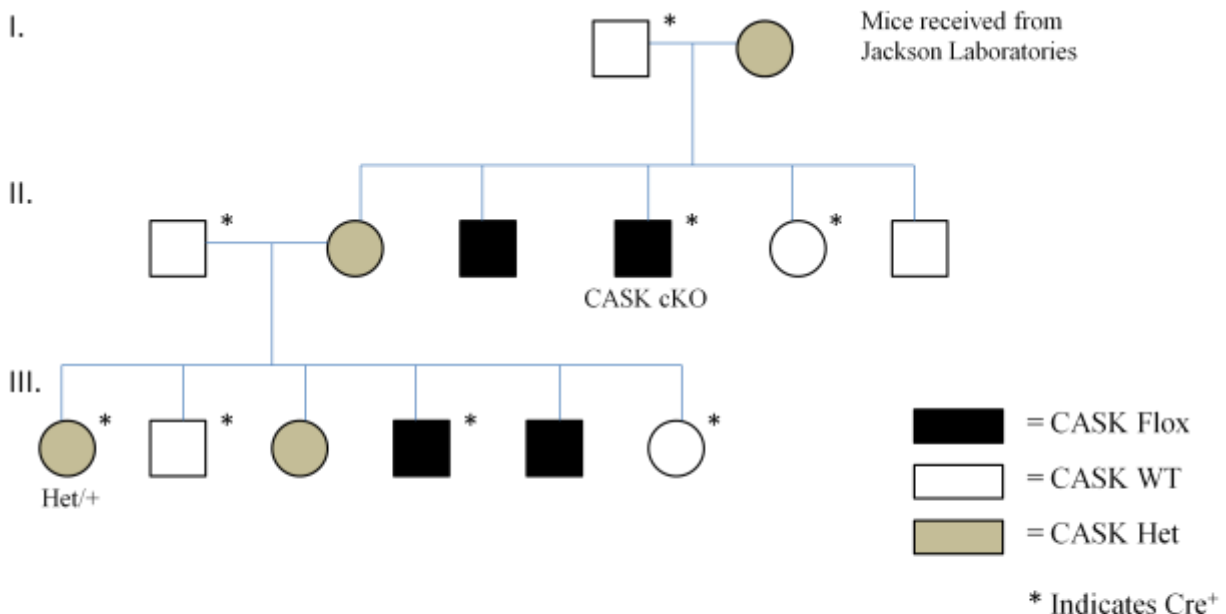


Figure 2-2: Mating scheme for CASK SkM cKO mice. (I) Mating of a WT Cre⁺ male with a CASK flox heterozygous female yields the F1 generation. (II) Matings were maintained by the same breeding scheme. (II) The F2 and every subsequent generation includes CASK cKO males and litter mate controls. Mendelian ratios predict that approximately 12.5% of the offspring will be CASK SkM cKOs.

CASK Ablation Occurs in the Cytoplasm but Not at the NMJ

To determine the efficacy of the HSA-Cre CASK skeletal muscle cKO model, western analysis of total skeletal muscle homogenates from 12 week old WT *Cre* transgenic positive (WT/+) and CASK cKO mice was performed (figure 2-3). Relative protein levels revealed that CASK expression had been almost entirely ablated in the CASK cKO muscle as compared to a strong band in the WT/+ homogenates. Verification of a loss of CASK protein expression in skeletal muscle was performed by western analysis on subcellular cytosolic fractions and membrane-enriched fractions (microsomes). These results revealed that CASK protein expression in total skeletal muscle homogenates was nearly absent, while CASK still remained expressed in lower levels in microsome membrane-enriched fractions. Due to the small amount of CASK still present in the membrane fraction via western analysis, we next conducted immunohistochemical analysis to verify these findings and determine the localization of the remaining CASK. Immunofluorescence was conducted on cross-sections of WT/+ and CASK SkM cKO quadriceps with a polyclonal CASK antibody. Confocal microscopy revealed that CASK expression had remained unaltered at the primary gutter of the NMJ in CASK cKO mice when compared to WT/+ littermate controls (figure 2-4). These results indicate that the HSA promoter coupled to Cre recombinase does not result in complete knockout of CASK expression in all skeletal muscle subcellular areas. Although CASK was not ablated completely from the NMJ, CASK protein levels in membrane-enriched fractions appeared reduced as compared to controls. This model, therefore, represents a cKO model in which CASK is lost from the cytosol and significantly reduced in the sarcolemma, but is still present at the NMJ.

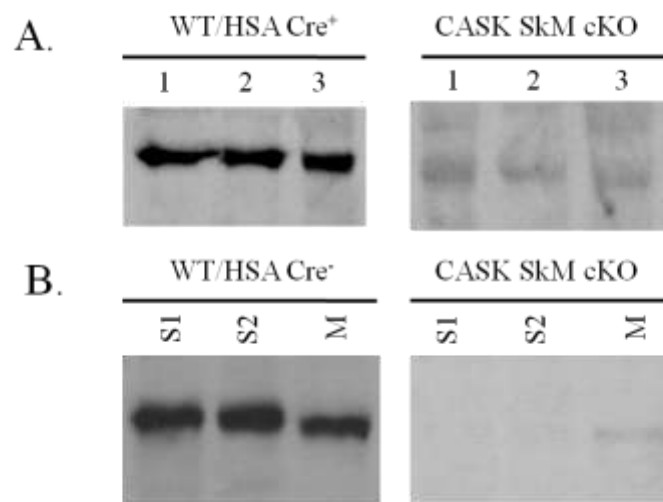


Figure 2-3: Western analysis of CASK protein expression in CASK SkM cKO mice. A.) Western analysis on total skeletal muscle protein homogenates from WT/+ and CASK SkM cKO mice confirmed that CASK protein expression is lost in CASK SkM cKO mice. B.) Western results on subcellular fractions demonstrated that CASK is missing in the cytosolic fractions (S1 and S2) of CASK SkM cKO skeletal muscle, but there is still a small amount of CASK present in the membrane enriched fraction (M). This data suggests that CASK SkM cKO mice may still be expressing CASK protein at the NMJ.

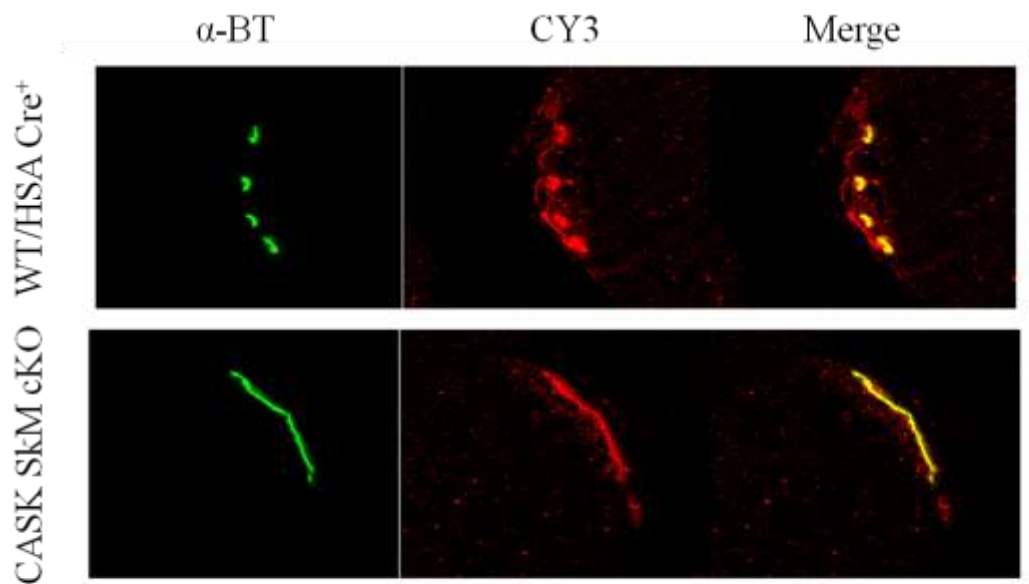


Figure 2-4: Confocal microscopy analysis of CASK protein subcellular localization in CASK SkM cKO mice. We conducted immunohistochemical analysis on fixed 8 μ m sections of quadriceps muscle using an affinity purified CASK polyclonal antibody (Zymed). Confocal analysis revealed that CASK protein expression at the primary gutter of the NMJ is not altered in CASK SkM cKO mice (bottom panel), as compared to WT/HSA Cre⁺ mice (top panel).

No Observable Degenerative Phenotype is Associated with a Loss of Non-Synaptic CASK

In order to assess the physiological effects of a loss of CASK from the cytoplasm of skeletal muscle fibers, we began to phenotypically characterize the HSA-CASK cKO mice to delineate any essential functions of non-synaptic CASK. Using this model, any characterized degeneration, loss of function, or altered levels and localizations of CASK protein interactors can be attributed to a loss of non-synaptic CASK. To begin the characterization of the CASK SkM cKO mice, morphological analysis was conducted via H&E staining on sections of WT/+ and CASK cKO quadriceps (figure 2-5). These results demonstrated that levels of inflammation and degeneration appeared relatively low in both CASK SkM cKO and WT/+ sections. There were also no observable differences in fiber size between the two genotypes. Newly regenerated muscle fibers have recently undergone degeneration and can serve as an indicator of recent skeletal muscle degeneration. Therefore, degeneration was quantified by counting the number of centrally nucleated fibers, a characteristic of a muscle fiber that has undergone degeneration and has been newly regenerated. There were no statistically significant differences in the number of centrally nucleated fibers between genotypes (data not shown).

Despite the observation that CASK ablation does not occur at the synapse, we chose to analyze the topology of the NMJ across genotypes. We postulate that cytosolic CASK may be involved in the trafficking of specific channels and receptors to the NMJ, and that there would be a loss of potential protein trafficking to the NMJ in the CASK SkM cKO mice when compared to WT mice, thus affecting its morphology. The topology of the NMJ was viewed for both WT/+ and CASK cKO mice. Fluorescent-conjugated α -bungarotoxin staining can be used to stain the primary gutter of the postsynaptic NMJ so that the morphology of the NMJ can be viewed, forming a “pretzel-like” architecture (figure 2-6). NMJ circumference was traced so that the

surface area could be measured and compared quantitatively across genotypes. Results showed no statistically significant difference between the sizes of the WT/+ and CASK cKO NMJs. There was also no observable difference in the amount of branching or continuity in NMJ topology across genotypes. Confocal analysis also revealed that candidate receptor and channel CASK interactors appeared normal at the NMJ.

To test for any weaknesses in force generation from CASKSkM cKO skeletal muscle, physiological experiments were conducted on dissected EDL muscles from WT/+ and CASK SkM cKO mice. By stretching the muscle and determining the length at which maximum isometric force is achieved, we can determine differences in muscle strength between genotypes. Results showed no discernible differences between WT and cKO mice, suggesting that muscle strength is not affected by a loss of cytosolic CASK in skeletal muscle (data not shown).

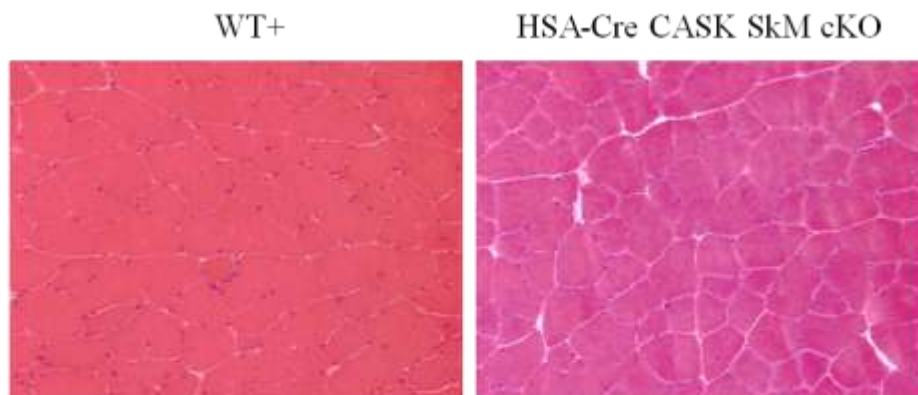


Figure 2-5: Histological analysis of HSA-Cre CASK SkM cKO mice. H&E staining of WT/+ and HSA-Cre CASK SkM cKO quadriceps demonstrated that levels of inflammation and degeneration appeared relatively low in both genotypes. There were also no observable differences in fiber size between the two genotypes. These results indicate that the HSA-Cre SkM cKO mice exhibit no histological phenotype.

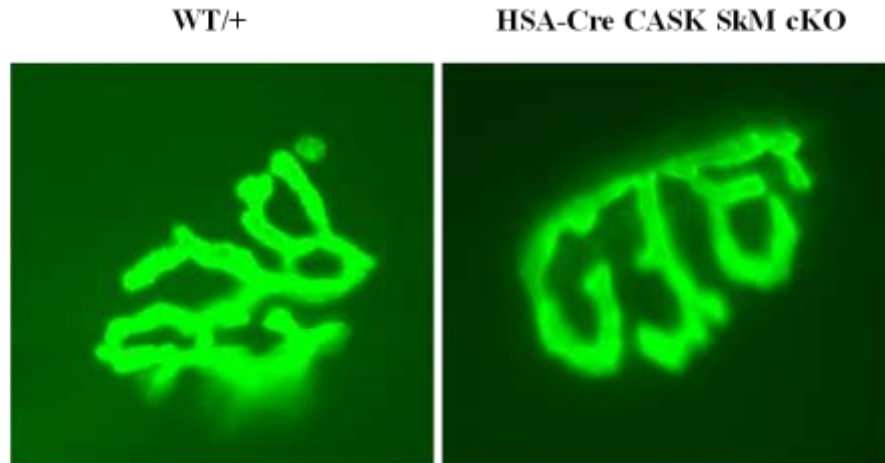


Figure 2-6: Epifluorescent analysis of the morphology of the NMJ. The topology of the NMJ can be viewed by staining longitudinally-sectioned quadriceps (30 μ m) with α -bungarotoxin. Results showed little differences between the NMJs from WT/+ and HSA-Cre CASK SkM cKO mice. Branching of the NMJ was relatively consistent across both genotypes, and there was no statistically significant difference between average NMJ surface area across the genotypes.

Loss of CASK Alters Dlg Protein Expression Levels and Subcellular Localization in CASK SkM cKO Mice

In addition to protein channels and receptors, CASK has also been shown to interact and bind with other MAGUK proteins *in vivo*. To determine if any MAGUK proteins were being upregulated in response to cytosolic CASK loss, we performed western analysis on CASK SkM cKO and WT subcellular fractions (figure 2-7). Because Dlg is already known to interact with CASK in skeletal muscle, we decided that Dlg would be the best protein candidate to analyze first. Western analysis demonstrates that Dlg protein levels in nuclear, total homogenate, cytosolic, and membrane fractions were all increased in CASK SkM cKO mice compared to their WT/+ littermates. Dlg upregulation may occur in response to a loss of cytosolic CASK due to the similar function they share in skeletal muscle.

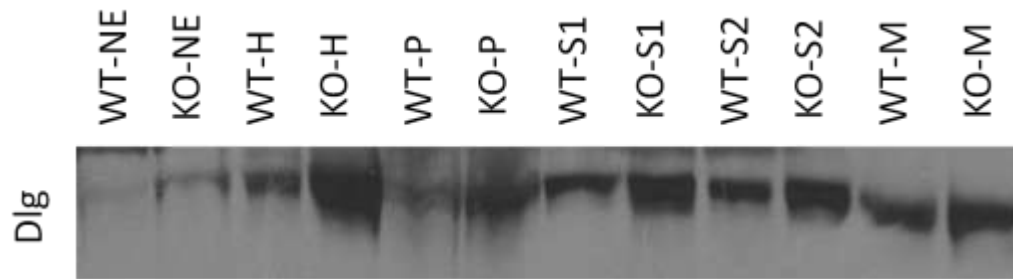


Figure 2-7: Dlg protein expression in subcellular fractions of WT/+ and HSA-Cre CASK SkM cKO mice. To determine if loss of CASK in skeletal muscle cytoplasm affected some of its various protein interactors, Western analysis was conducted on NE (pure nuclear extract), H (total skeletal muscle homogenate), P (crude nuclear pellet, containing nuclei, mitochondria, lysosomes), S1 (cytosolic fraction containing ribosomes, golgi and membrane proteins), S2 (soluble cytosolic fraction) and M (membrane-enriched fraction) fractions from WT/+ and cKO total skeletal muscle. Westerns conducted with a monoclonal Discs large (Dlg) antibody (UC Davis) indicate that Dlg protein expression may be upregulated in HSA-Cre CASK SkM cKO mice as compared to WT muscle.

As a means of confirming the Dlg Western analysis and analyzing the subcellular localization of the excess Dlg in CASK SkM cKO mice, we performed immunohistochemical analysis on fixed 8 μ m quadriceps cross-sections from WT/+ and CASK SkM cKO mice. Confocal microscopy revealed that excess Dlg was found localized to the cytoplasm and at membranes in cKO mice when compared to WT/+ mice, while similar localization patterns were seen at the NMJ in both genotypes (figure 2-9). This further supports the role that Dlg upregulation occurs to compensate for the loss in function that arises from ablation of cytoplasmic CASK.

Since Dlg was being upregulated, we also wanted to know if any other MAGUK protein levels were being altered in response to a loss of non-synaptic CASK. To determine this, we performed western analysis on WT/+ and HSA-Cre CASK SkM cKO subcellular fractions using a Pan-MAGUK antibody that recognizes a conserved MAGUK consensus domain (figure 2-8). This analysis illustrated that MAGUKs other than Dlg may also be altered in skeletal muscle in response to a loss of cytoplasmic CASK.

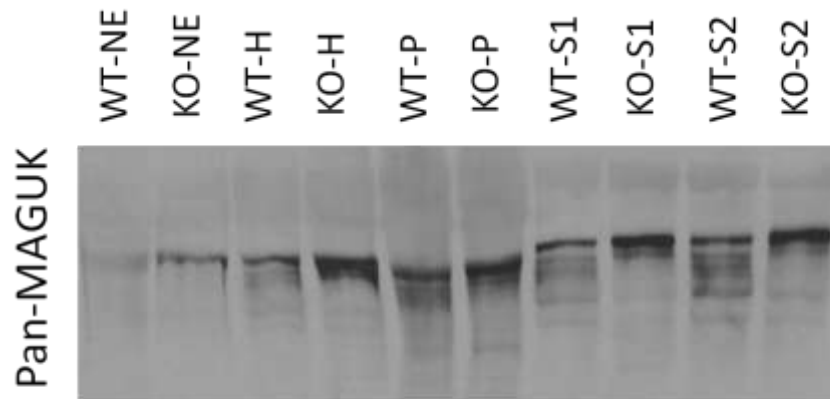


Figure 2-8: MAGUK protein expression in subcellular fractions of WT/+ and HSA-Cre CASK SkM cKO mice. To determine if loss of CASK in skeletal muscle cytoplasm affected other MAGUK protein levels, Western analysis was conducted on NE, H, P, S1, and S2 fractions from WT/+ and cKO total skeletal muscle. Westerns conducted with a Pan-MAGUK antibody indicate that MAGUK protein expression may be altered in HSA-Cre CASK SkM cKO mice as compared to WT muscle.

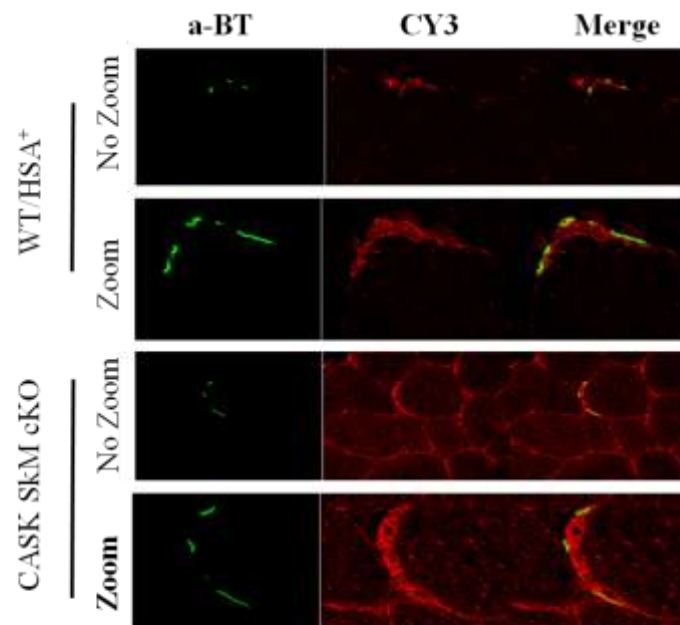


Figure 2-9: Confocal analysis of Dlg subcellular localization in HSA-Cre CASK SkM cKO mice. Dlg localizes to the membrane and NMJ, and is upregulated in the cytoplasm in CASK SkM cKO mice. However, localization is not altered between WT/+ and cKO mice.

We next examined the localization of Glutamate Receptor Interacting Protein (GRIP), another PDZ containing protein and known CASK interactor in skeletal muscle (figure 2-10) (Sanford, personal communication). GRIP is responsible for binding glutamate receptor subunits, and is composed of 7 PDZ domains. Immunohistochemical analysis, however, revealed that there were no major differences in GRIP localization between WT/+ and CASK SkM cKO mice. This shows that a loss of non-synaptic CASK does not alter levels of the CASK protein interactor GRIP.

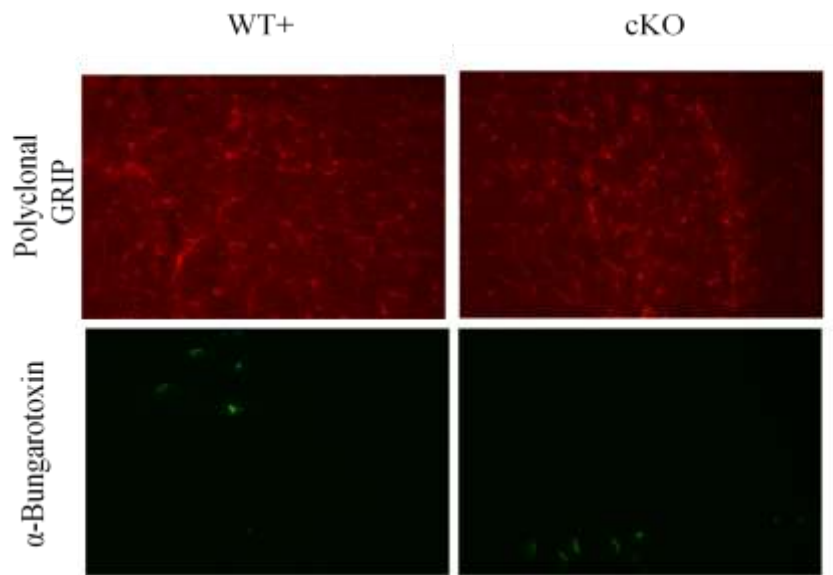


Figure 2-10: Immunofluorescence analysis of the localization of the GRIP protein in CASK SkM cKO skeletal muscle. Immunofluorescence analysis was conducted on unfixed 8 μ m sections of 12-week old WT and cKO quadriceps. Epifluorescent microscopy revealed that GRIP expression at the cytoplasm or fiber membrane remained unaltered between WT and CASK SkM cKO skeletal muscle.

Discussion

In this study, we hoped to test the hypothesis that a complete ablation of CASK from skeletal muscle would result in an observable phenotype. However, with the HSA-Cre model, CASK was not completely knocked out of skeletal muscle. In fact, CASK protein expression had been knocked out in the cytoplasm, but remained expressed at the NMJ. This model, however, did provide us with a means to differentiate a function between cytoplasmic CASK and CASK present at the NMJ.

In the current HSA-Cre model, we found that cytoplasmic CASK is involved in the levels and localization of other protein interactors, and a knockout of CASK in the cytoplasm results in alterations in these levels. However, no other discernible phenotype could be noted. There were no signs of abnormal skeletal muscle maintenance and development, nor were there any signs of histological abnormalities or muscle degeneration.

A likely explanation for the lack of phenotype in the CASK SkM cKO model is the probability that CASK and Dlg share similar functions in skeletal muscle (Nix *et. al.* 2000). As seen in KO models of CASK and Dlg, when function of either protein is lost, both models exhibit similar debilitating phenotypes, followed by death within 24 hours (Lavery *et. al.* 1998, Caruana *et. al.* 2001). This suggests that CASK and Dlg may be implicated in the same developmental pathway in skeletal muscle. The upregulation of Dlg seen in the CASK SkM cKO mice may compensate for the loss of cytosolic CASK function, thus preventing the characterization of an observable phenotype. The altered expression levels of MAGUK proteins between CASK SkM cKO and WT/+ protein homogenates seen through Western analysis may also suggest the possibility of other MAGUK proteins that share redundant functions with

CASK. These results demonstrate the importance of the interaction of CASK and Dlg, as well as the function of MAGUK proteins in skeletal muscle development.

The lack of CASK ablation at the NMJ in the HSA-Cre CASK cKO may be due to our choice of promoter. The HSA promoter was chosen for its availability and selectivity for skeletal muscle. Miniou *et al* had previously created the HSA-Cre line of mice, and they were available for purchase through Jackson Laboratories. Since then, a number of other studies had made use of the Cre/lox system under the direction of the HSA promoter, yielding skeletal muscle specific knockout models. The HSA-Cre system was designed to be expressed in skeletal muscle starting at approximately e9; however expression of the system in synaptic nuclei, the nuclei in skeletal muscle located directly beneath the neuromuscular junction, has not been verified. Synaptic nuclei specifically express other genes whose products are specifically localized to the post-synaptic membrane, such as acetylcholine receptors and utrophin (Grady *et. al.* 2005, Jevsek *et. al.* 2006, Kummer *et. al.* 2006, Schaeffer *et. al.* 2001). It is possible that CASK is effectively knocked out throughout cytoplasm, but still getting transcribed in synaptic nuclei and being specifically trafficked into the NMJ. To account for this, we could create a model in which Cre recombinase expression was driven under the direction of a different promoter that was known to be expressed within synaptic nuclei. However, no such Cre transgenic line has yet been made available.

Future experiments with the HSA-Cre CASK SkM cKO mice may include the isolation of myofibers in cell culture, which will provide us with the opportunity to further examine the effects on CASK protein interactors with the ablation of non-synaptic CASK. Microarray analysis can also be performed across the different genotypes, allowing us to determine which genes may be more or less transcribed in response to a loss of non-synaptic CASK. 2-

dimensional gel proteomic analysis would be able to provide us with more complete data about post-transcriptional modification or altered expression levels of CASK protein interactors. To verify that CASK is still getting transcribed in synaptic nuclei in the HSA-Cre CASK SkM cKO model, *in situ* hybridization can be performed to determine the presence of the *lox P* flanked exon 1 of the CASK gene in synaptic nuclei. A collaborator is currently developing an acetylcholinesterase promoter-driven Cre in an adeno-associated viral (AAV) vector that we eventually hope to test in 4-week old HSA-Cre CASK SkM cKO mice. This will knock out the remaining synaptic CASK after development to determine CASK function at the NMJ in adult skeletal muscle after development. However, to better understand the functions of CASK in developing skeletal muscle, a more complete CASK cKO model must be constructed in which Cre recombinase is under the direction of a skeletal muscle-specific promoter that is expressed earlier in development so that CASK deletion is present in synaptic nuclei.

III

Characterization of Total CASK Function in Skeletal Muscle

Introduction

CASK has been shown to be involved in protein trafficking and receptor clustering in neuronal cultures (Atasoy et. al. 2007, Butz et. al. 1998, Daniels et. al. 1998, Hong et. al. 2006, Hsueh et. al. 1998, Hsueh et. al. 2006, Jeyifous et. al. 2009, Kim et. al. 1995, Kornou et. al. 1997, Leonoudakis et. al. 2004, Maximov et. al. 1999, Samuels et. al. 2007). The presence of CASK in skeletal muscle has led to the hypothesis that CASK may play a similar role here in trafficking proteins to the NMJ. We explored this hypothesis in our previous aim by looking for the presence of a degenerative phenotype associated with a loss of non-synaptic CASK. However, the localization of CASK to the NMJ suggests that CASK may also play a significant role at the junction of the skeletal muscle with the motor neuron. To determine the role that CASK plays at the NMJ, a complete cKO model must be generated in which CASK is not only lost from the cytoplasm and reduced in the peripheral membrane, but completely absent in skeletal muscle tissue. Using this model, it will be possible to gain a broader insight into the specific mechanisms by which CASK functions *in vivo* in skeletal muscle.

To further delineate the mechanisms by which CASK functions at the NMJ, our lab generated cKO models of CASK ablation in the cytoplasm, membrane, and NMJ of murine skeletal muscle. Once again, the Cre/lox recombination system was used to interrupt the CASK gene by selectively excising exon 1 (Atasoy et. al. 2007). The *Cre* transgene in this model is expressed under the direction of the myogenic factor 5 (Myf5) promoter. Myf5 is a myogenic regulatory factory, involved in the differentiation and development of mature skeletal muscle (Buckingham et. al. 2003, Brand-Saberi 2005, Perry and Rudnick 2000, Rescan 2001, Shih et. al.

2008). It becomes expressed early in development before NMJ formation and the specialization of synaptic nuclei. To achieve skeletal muscle specific ablation, mice expressing the Myf5-Cre transgene were bred to mice that contain the floxed CASK allele generated by Atasoy *et al.* (Atasoy *et. al.* 2007). Generation of a more complete CASK SkM cKO model will allow for a more comprehensive analysis of the role of CASK function in skeletal muscle and at the NMJ.

As we have previously demonstrated, a loss of non-synaptic CASK protein expression does not result in any gross, morphological, or functional phenotypes. However, it has been shown that a complete ablation of CASK results in neonatal lethality in mice (Lavery *et. al.* 1998). Given this data, we postulate that CASK may play a more crucial role in muscle development and at the mature NMJ, and that a loss of synaptic CASK will result in a pathological phenotype. We hypothesize that CASK may be involved in synaptic scaffolding and its role in muscle is essential for normal function and development.

Methods

The Myf5-Cre Transgenic Line and CASK Knockout Models

The Cre/lox method of gene excision was once again used for the generation of the new CASK SkM cKO model. CASK floxed mice, generated by the Sudhof laboratory and used in the creation of the HSA-Cre CASK SkM cKO model, were purchased from Jackson laboratories. Mice that contain a *Cre* expression cassette knocked into the muscle-specific Myogenic factor-5 (Myf5) promoter were used to generate tissue-specific CASK ablation at an earlier point in muscle development. Myf5 is a myogenic regulatory factor expressed in developing skeletal muscle. In order to generate the new CASK SkM cKO mice, female CASK floxed heterozygous mice were mated to male Myf5-Cre mice to create male progeny that contain both the floxed

CASK allele and the Myf5-Cre allele. Because CASK is X-linked, these mice are predicted to have a complete ablation of CASK in skeletal muscle tissue starting by day e8 in development when Myf5 is normally expressed in developing myotubes (cKO). The progeny from this cross also includes wild type littermates that either contain or lack the Myf5-Cre transgene (WT/+ and WT/-, respectively). Flox/- males and Het/- females are also present in this generation.

All mice are housed in a fully-equipped vivarium at The Ohio State University under an IACUC approved protocol to Dr. Jill Rafael-Fortney. Mice are kept on a standard 12 hr day/night cycle and were provided food (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories) and water *ad libitum*.

Genotyping

Genomic DNA was obtained from the tail of 3 week-old mice at weaning or from the tail, head, or placenta of mice removed prenatally from pregnant females and analyzed via PCR. To test for the presence of the Myf5-Cre transgene, the previously described HSA-Cre F/R primers by Miniou *et al* were used to generate a 790 bp product from within the *Cre* region. Genotyping for presence of the floxed allele was performed using the two sets of primers previously described in Chapter 2. The first primer set, CASK Neo Rejuv F/R, was designed to screen for a region within the *Neo* cassette, yielding a 610 bp product. The second primer set was used in conjunction with CASK Neo Rejuv F/R to confirm the presence of the floxed allele, and are as follows: IMR 6389, located within the *Neo* cassette and IMR 6390, located within the CASK genomic sequence, yielding a 256 bp product. To test for the presence of the wild type allele, the primer pair SSC 221 and IMR 6388 was designed to screen for a DNA sequence around the *Neo* cassette of the targeting sequence generated by the Sudhof lab (primers designed by Jackson

Labs). As previously described, these primers yielded a 490 bp product for the WT allele and a 1,400 bp product for the floxed allele. All PCR reaction products were run on 1% agarose gels (0.02% ethidium bromide) and photographed under ultraviolet light.

Histological Analysis

Unfixed total hindlimbs were dissected from entire litters of prenatal mice (e12-20) and embedded in OCT (optimal cutting temperature) cryoprotective mounting medium (Tissue-Tek, Hatfield, PA). Because of the small size of the mice, the entire leg was used to maintain the integrity of the skeletal muscle tissue. Tissues were then slow-frozen on liquid nitrogen-cooled isopentane and stored at -80°C. Frozen tissue was cut into 8 µm cross-sections on a cryostat. To view the histology of the skeletal muscle tissue, slides were fixed in 100% ethanol and stained with hematoxylin and eosin (H&E) using a standardized protocol.

Immunohistochemistry

Immunohistochemical analysis was conducted on 8 µm cross-sections of unfixed frozen legs from WT^{-/-}, WT^{+/+}, Flox, and CASK cKO prenatal mice (e12-20). Sections were first blocked in 1% gelatin in potassium-phosphate buffered saline (KPBS) for 15 min before being washed in KPBS for 15 min. Following the washes, sections were incubated for 2 hours in a primary antibody solution consisting of anti-dystrophin/utrophin (1:10,000) diluted in KPBSG (KPBS + 0.5% gelatin) plus normal goat serum (1%). Sections were then washed 3 x 5 min in KPBSG. Following rinsing, sections were incubated in a secondary antibody solution that consists of CY3 anti-rabbit secondary antibody (Jackson Laboratories, 1:200), Alexa 488-conjugated α -bungarotoxin (Molecular Probes, Inc., 1:1000), and normal goat serum (1%) in

KPBSG for 1 hour. α -bungarotoxin was used to fluorescently label the acetylcholine receptors at the NMJ. After incubation, sections were once again washed in KPBSG 3 x 5 min. Slides were then mounted in Vectashield® (Vector Labs, Burlingame, CA) and DAPI (500 ng/mL) before being coverslipped. Images were captured with a Nikon Eclipse E800 microscope and a SPOT-RT slider digital camera and SPOT® software.

Results

Generation of Myf5-Cre CASK SkM cKO Mice

The Myf5-Cre CASK SkM cKO mice were again generated via the Cre/lox recombination system. Mice that possess the floxed CASK allele were mated to mice that express the *Cre* transgene from the Myf5 promoter to interrupt the CASK gene specifically in skeletal muscle tissue, creating a generation of mice that include: CASK SkM cKO males, flox/- males, het/+ females, het/- females, and WT/+ and WT/- mice (refer back to figure 2-2).

Myf5-Cre CASK SkM cKO Mice are Not Born at Mendelian Ratios

Under the aforementioned breeding scheme, few mice were determined to be CASK SkM cKO mice at weaning when compared to littermates. Observed Mendelian ratios were not as expected, and there were very few viable CASK SkM cKO mice (figure 3-1). As this trend was observed, daily observations of matings were initiated in an attempt to catch any dying pups. Four dead pups were genotyped as cKOs but sections could not be collected due to the deterioration of the samples. No dead pups were observed after 48 hours after birth. To account for the possibility that CASK SkM cKO mice may be dying prior to or at birth, Caesarean

sections were performed on Het/- female mice after mating with Myf5-Cre males to obtain DNA for genotyping and quadriceps cross-sections for histological analysis.

Caesarean sections were performed on mice approximately 16.5 days following fertilization. Timed matings were set up to determine the exact day that fertilization took place. At e16.5, prenatal mice were dissected to obtain tissue for DNA extraction and staining. Because of the small size of the prenatal mice, individual quadriceps muscle could not be isolated without sacrificing the integrity of the tissue. To obtain sections for staining, both legs were removed and placed in OCT to be frozen and cut.

<i>Genotype:</i> Males (31 total)	CASK wt/Y; Myf5-Cre Tg+	CASK wt/Y; Tg-	CASK Flox/Y; Myf5-Cre Tg+ (cko)	CASK Flox/Y; Tg-
<u>Expected:</u>	7	8	8	8
<u>Observed:</u>	7	14	2	8
<i>Genotype:</i> Females (36 total)	CASK wt/wt; Myf5-Cre Tg+	CASK wt/wt; Tg-	CASK Flox/wt; Myf5-Cre Tg+	CASK Flox/wt; Tg-
<u>Expected:</u>	9	9	9	9
<u>Observed:</u>	11	15	2	8

Figure 3-1: Observed and expected Myf5-Cre CASK SkM cKO mice genotypes based on Mendelian genetics. There were fewer CASK cKO males and Het/+ females observed than what was predicted by Mendelian genetics, while other observed genotypes lie very close to expected values. This suggests that cKO males and Het/+ females may be dying *in utero*.

Myf5-Cre CASK SkM cKO Mice May Exhibit a Degenerative Phenotype

Myf5-Cre CASK SkM cKO mice were removed from the pregnant female at e16.5 and dissected for tissue. Upon dissection, Myf-5-CASK cKO mice appeared smaller and underdeveloped when compared to the rest of the litter. Histological analysis on these mice reveals an apparent muscle pathology when compared to WT/+ littermate controls. The muscles in the hindlimb of the cKO mice were less developed than those of the littermates at the same age and were smaller in circumference than wild-type mice. Muscle fibers were less defined and fiber membranes appeared relatively uneven in the Myf5-CASK cKO mice. This suggests that a complete loss of CASK during development results in abnormal skeletal muscle myogenesis.

To determine the extent to which development has slowed, cKO hindlimbs were immunostained with an antibody that binds dystrophin and utrophin, two skeletal muscle fiber markers that are localized around developing and mature skeletal muscle fiber membranes and used to detect differentiated muscle fibers. Dystrophin staining revealed that the Myf5-CASK cKO mice appear to have much less differentiated muscle than WT/+ littermates. Co-staining with the fluorescent nuclear dye DAPI also reveals a high degree of disorganization among the skeletal muscle fibers in the Myf5-CASK cKO hindlimb as compared to littermate controls. This data suggests that synaptic CASK plays an essential role in myogenesis.

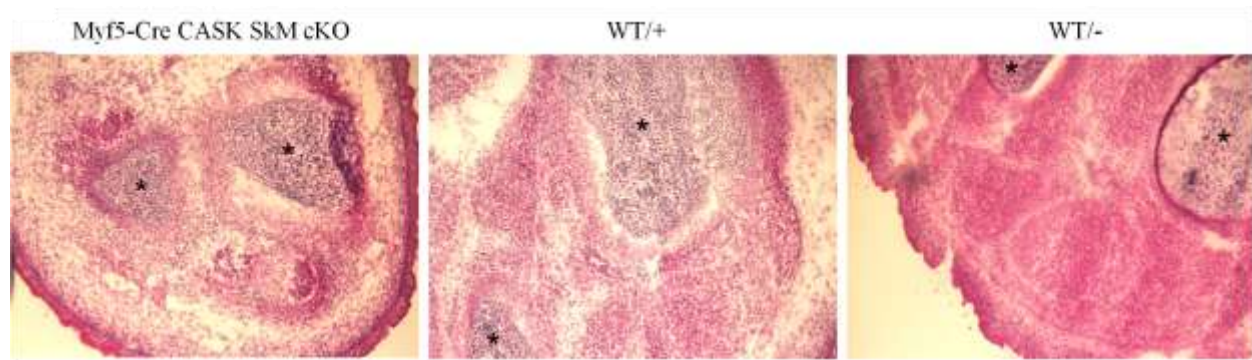


Figure 3-2: Histological analysis of Myf5-Cre CASK SkM cKO mice. H&E stained sections of hindlimb from mice at e16.5 reveals that the Myf5-Cre CASK SkM cKO mouse appears to have less well developed skeletal muscle between and surrounding the bones of the lower hindlimb when compared to WT/+ and WT/- littermate controls. The purple staining bones are each marked with an *. All panels are the same magnification.

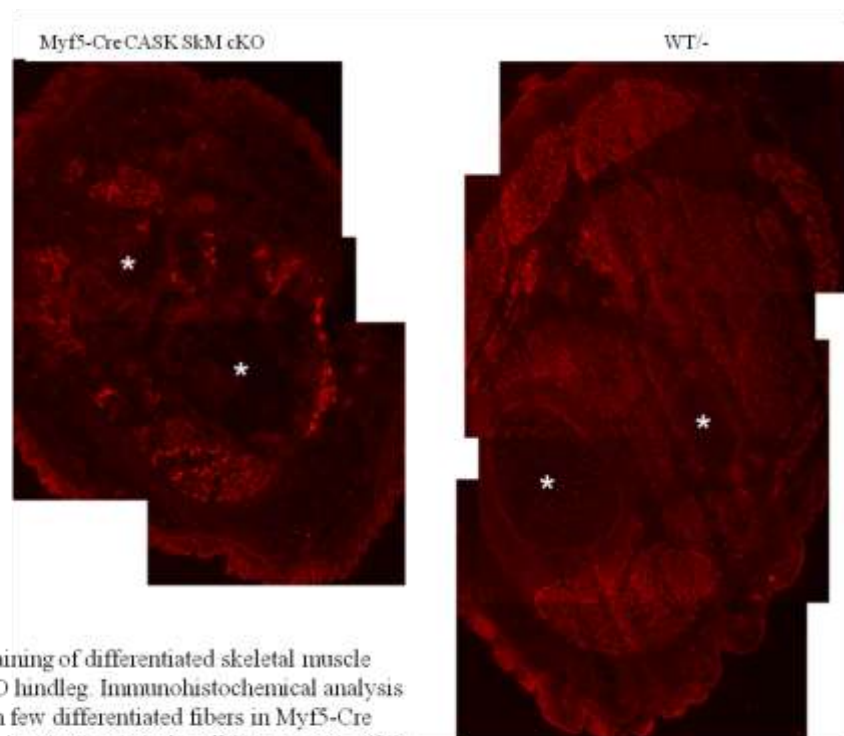


Figure 3-3: Dystrophin/utrophin staining of differentiated skeletal muscle fibers in Myf5-Cre CASK SkM cKO hindleg. Immunohistochemical analysis reveals a less organized muscle with few differentiated fibers in Myf5-Cre CASK SkM cKO mice compared to littermate controls. All images were taken at the same exposure and are at the same magnification.

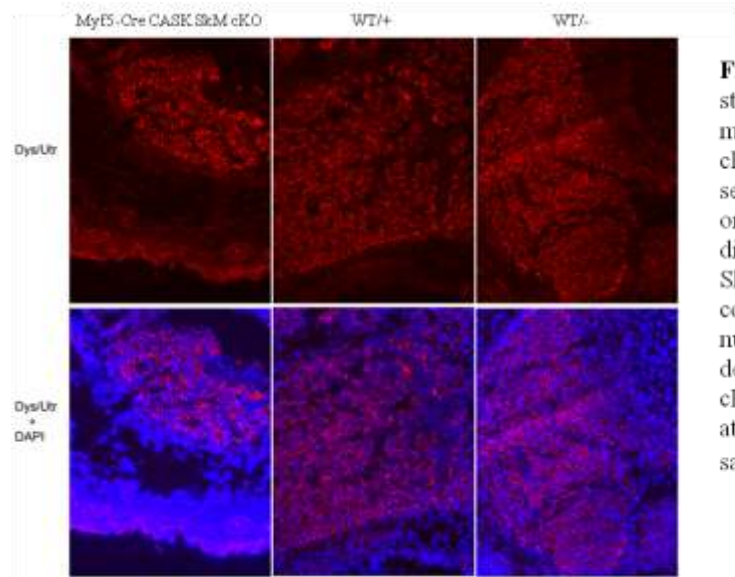


Figure 3-4: Dystrophin/utrophin staining of differentiated skeletal muscle fibers in Myf5-Cre CASK SkM cKO hindlimbs. Staining of these sections from mice at e16.5 show a less organized muscle with few differentiated fibers in Myf5-Cre CASK SkM cKO mice compared to littermate controls (red). Co-staining with the nuclear stain DAPI (blue) further demonstrate the disorganization of the cKO hindlimbs. All images were taken at the same exposure and are at the same magnification.

Discussion

In this study, we hoped to develop a more complete CASK SkM cKO model in which CASK was completely ablated throughout skeletal muscle tissue. Unlike the previous model in which only non-synaptic CASK was lost, Cre is under the direction of the Myf5 promoter, which should be expressed earlier in development at e8. Myf5 is the first of the myogenic regulatory factors to be expressed and it is expressed in the epaxial lip of the dermomyotome (Tajbakhsh et al. 1996). This allows for the complete interruption of the CASK gene in all nuclei so that there won't be any protein expression or subcellular localization of CASK within skeletal muscle tissue.

Our first observation with the Myf5-Cre CASK SkM cKO mice was the low number of mice that exhibited both the floxed CASK allele and expression of the Cre transgene. Compared to expected numbers of mice as predicted by Mendelian genetics, there were few observed

CASK SkM cKO mice born. This piece of evidence led us to the hypothesis that Myf5-Cre CASK SkM cKO mice may be dying *in utero* or very soon after birth. To account for this observation, we began performing Caesarian sections on female Het/- mice with litters on e16.5. Initial gross observation of the dissected prenatal mice revealed a potential phenotype, as cKOs were smaller than their WT littermates. Morphological analysis confirmed that the skeletal muscle fibers of the Myf5-Cre CASK SkM cKO mice were less organized and developed around the bones of the lower hindlimb. These findings were confirmed when immunostaining with an anti-dystrophin antibody revealed that there were fewer organized, differentiated muscle fibers in the Myf5-Cre CASK SkM cKO mice when compared to WT littermate controls. These results suggest that synaptic CASK is essential for normal muscle development.

Further phenotypic analysis of this mouse model will be necessary to determine the role that CASK has on muscle development. As done with the HSA-Cre CASK SkM cKO mouse model, future work involves western analysis to confirm that CASK has indeed been completely knocked out from skeletal muscle tissue and immunohistochemical analysis with a CASK antibody to confirm. Functional analysis can be conducted as well to determine any muscle weakness that results from a loss of CASK in skeletal muscle. In addition, immunohistochemical and western analysis can be performed to determine the effects a complete loss of synaptic CASK has on the levels and localizations of other CASK protein interactors. NMJ localization can be viewed by co-staining with α -bungarotoxin to determine any differences in the localization of other specific proteins recruited to the NMJ early in development between cKO mice and their littermate controls of the same age. Proteomic analysis can also be conducted on skeletal muscle homogenates from these mice to determine which proteins are being affected by a loss of CASK and how.

Additionally, future experiments can be conducted to further the explanation of why a degenerative phenotype is occurring. Transmission electron microscopy can be conducted between cKO and WT mice to examine if sarcomerogenesis is affected. Also, we can determine if the phenotype is caused by altered proliferation through Ki67 staining, apoptosis through ApoTaq detection, or fiber number or MHC expression. Furthermore, if the Myf5-CASK cKO mice show that CASK is involved in myogenesis, we can examine embryonic myogenesis in a murine model that overexpresses the CASK full-length protein (Sanford et. al. 2004) to determine if this increases the rate of myogenesis. The research being conducted on this aim is in its early stages and there is still much to be done. However, the finding that there may be a potential degenerative phenotype associated with a complete ablation of CASK in skeletal muscle will drive further study of the CASK protein.

IV

The Role of CASK as a Protein Kinase and Its Phosphorylation

Introduction

CASK contains two protein domains that share similar sequence homology to functional kinases and have led to the hypothesis that CASK may play a role in protein phosphorylation: the guanylate kinase (GK) domain and the Ca^{2+} /calmodulin kinase-like (CaMK) domain. The GK domain is found at the C-terminus end of CASK and is named for its sequence similarity to the yeast guanylate kinases. However, the GK domain found in MAGUKs lacks the amino acid residues for binding ATP, and thus is predicted to not possess any actual phosphorylative ability (Kuhlendahl *et. al.* 1998, McGee and Bredt 1999, Olsen and Bredt 2003). Instead, the primary functions of the GK domain are to confer scaffolding properties to CASK via its interactions with the SH3 domain of other MAGUK proteins, as well as participate in intramolecular interactions with its own SH3 domain (MacLennan and Edwards 1990, McGee and Bredt 1999, McGee *et. al.* 2001, Nix *et. al.* 2000, Shin *et. al.* 2000, Tavares *et. al.* 2001). The CaMK domain is completely unique to CASK and was named for its similar sequence homology to the CaMK family of proteins, notably, CaMKII (Hata *et. al.* 1996). CaMKII is a serine/threonine specific protein kinase known to be regulated by the Ca^{2+} /calmodulin complex. CaMKII has been shown to phosphorylate both AMPA and NMDA receptor subunits in central nervous system synapses, including the subunits GluR1 and NR2B. The phosphorylation of these glutamate receptor subunits has been postulated to play a major role in synaptic plasticity and long term potentiation. The association of CaMKII with MAGUK proteins has been shown to both enhance its catalytic activities and to phosphorylate MAGUKs such as PSD-95 and Dlg (Song *et. al.* 2004, Yan *et. al.* 2004, Yoshimura *et. al.* 2000 and 2002).

Despite the similar sequence homology of the CaMK domain to CaMKII, it had not been believed to function similarly. Due to its lack of Mg^{2+} -binding sites, the CaMK domain had been classified as a catalytically inactive pseudokinase and has been previously thought to be ineffective (Boudeau *et. al* 2006). However, recent evidence has shown that the CaMK domain adopts a catalytically active conformation that binds ATP without Mg^{2+} and catalyzes phosphotransfer to neurexin-1, itself, and possibly other CASK protein interactors. Neurexin-1 is a synaptic cell-adhesion protein that binds CASK by means of its PDZ domain (Ichtchenko *et. al.* 1996, Ushkaryov *et. al.* 1992). The CASK-neurexin complex has been shown to be a substrate for phosphorylation by the CaMK domain in neuronal cultures. CASK is unique in that it is the first known protein of its kind to be catalytically active in the absence of Mg^{2+} (Mukherjee *et. al.* 2008). This allows CASK to function faster in the Mg^{2+} -poor environment of developing skeletal muscle where other kinases catalyze more slowly, making CASK a very important protein to investigate as an active kinase in developing skeletal muscle.

Interestingly, there is much evidence that shows that the CASK interactor Dlg is phosphorylated and that this phosphorylation regulates its activities at synapses (Massimi *et. al.* 2006, Gardner *et. al.* 2006, Zhang *et. al.* 2007). At the *Drosophila* NMJ, Dlg has been shown to be phosphorylated by the PAR-1 kinase at a conserved serine residue in the GK domain. This phosphorylation was shown to regulate the trafficking of Dlg between extrasynaptic and synaptic compartments. Furthermore, phosphorylation of Dlg by PAR-1 plays a major role in the development of and synaptic transmission at the *Drosophila* NMJ (Zhang *et. al.* 2007). Recent evidence has shown that CaMKII phosphorylates both the L27 and PDZ domains of Dlg as well. This phosphorylation ultimately aids in the regulation of GluR1 receptor subunits at synapses (Nikandrova *et. al.* 2010).

While CASK may possess the ability to function as an active protein kinase, it also may play an important role as a protein substrate for phosphorylation. CASK has been shown to be phosphorylated by multiple proteins, including protein kinase A (PKA) and cyclin-dependent kinase 5 (Cdk5) (Huang *et. al.* 2010, Samuels *et. al.* 2007). Phosphorylation of CASK by PKA causes an upregulation of the interaction between CASK and the TBR-1 transcription factor. This enhanced interaction ultimately results in increased expression of the NMDA receptor subunit, NR2B (Huang *et. al.* 2010). Perhaps the most striking evidence for the need for investigation of phosphorylation of CASK has come from studies of the phosphorylation of CASK by Cdk5. CASK has been shown to be phosphorylated by Cdk5, the only member of a family of proteins that is not involved in the regulation of the cell cycle. When Cdk5 is absent *in vivo*, CASK is no longer phosphorylated in a central nervous system model. Furthermore, when Cdk5 is interrupted, aberrant neuronal synapse formation is present. Cdk5 deficient mice show a significant reduction in CASK expression at cell membranes, which has been directly attributed to a lack of phosphorylation of CASK, as shown by mutational analysis of its serine residues (Samuels *et. al.* 2007). Mice that are deficient in Cdk5 also demonstrate aberrant motor axon projection and acetylcholine receptor clustering (Fu *et. al.* 2005). Taken together, these results suggest that investigating the phosphorylation of CASK in skeletal muscle will likely provide valuable insight into the role of CASK in synaptic function and the tissue as a whole.

While the CaMK domain is capable of kinase activity in neuronal cultures, there is no data as to the degree to which the CaMK domain acts as a functional protein kinase in skeletal muscle. Our HSA-Cre CASK SkM cKO model represents a loss of non-synaptic CASK in skeletal muscle, so we turned to this model to determine the extent to which CASK phosphorylation and kinase activity regulate normal muscle development and function. In

addition, we looked to two other murine models in which CASK expression was altered. One model overexpresses the full-length CASK protein (CASK FL+) and the other overexpresses a truncated version of the CASK protein (CASK Δ+) (Sanford *et. al.* 2008). These models allow us to examine the extent to which CASK phosphorylation and kinase activity regulate normal muscle development and function in skeletal muscle. Dlg has been shown to be both a substrate for phosphorylation *in vivo* and a known CASK protein interactor, so we investigated the interaction between CASK and Dlg *in vitro* as well (Massimi *et. al.* 2006, Gardner *et. al.* 2006, Zhang *et. al.* 2007). We hypothesize that CASK functions as an active protein kinase in skeletal muscle and is involved in the phosphorylation of Dlg and other CASK protein interactors. In addition, we hypothesize that phosphorylated CASK may also play an essential role in the formation of the neuromuscular junction.

Methods

Immunoprecipitations

Immunoprecipitations were performed using total skeletal muscle homogenates from 12-wk old HSA-Cre CASK SkM cKO mice and their littermate controls in Newcastle Buffer. Skeletal muscle homogenates (30 μL) were preadsorbed with protein G sepharose (Amersham Biosciences) for 1 hour. The supernatant was then incubated with 13 μL of monoclonal CASK antibody (Chemicon) for ≥3 hours. The mixture was next incubated with protein G sepharose overnight. The protein-bead suspension was then spun down and the supernatant was kept as the unbound fraction. Beads were washed three times and boiled in 30 μL of Laemmli dye to release bound material, which was then run on an 8% SDS-PAGE gel and immunoblotted with anti-Cdk5.

Protein Isolation and Purification

E. coli BL21 cells were transformed with a pGEX plasmid vector containing either the Dlg or the CASK cDNA sequence linked to the glutathione-S-transferase (GST) fusion protein gene using a standardized heat-pulse method (Stratagene). Cells were then grown at 37°C in 2XYT media + ampicillin (75µg/mL) to a dilution of 1:100. The cells were allowed to grow an additional 3 hours before induction of GST-CASK or GST-Dlg transcription with isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) for 4-5 hours. Cells were then spun down for 10 min at 5,000 x g and resuspended in 1/20 their original volume in phosphate-buffered saline (PBS). Cells were lysed and spun down at 10,000 x g for 5 min at 4°C to separate soluble proteins from cellular debris. The supernatant was mixed with prepared glutathione beads (1/1,000 original volume of cells) and incubated overnight at 4°C. Following incubation, cells were spun down at 1,000 x g for 5 min to sediment the matrix. The bead pellet was washed three times in cold PBS. The fusion protein was then eluted by adding 1 mL of 50 mM Tris-Cl, pH 8.0, 5 mM reduced glutathione solution. The eluate fraction was then isolated and stored at -80°C. Fractions from the pre-induction, post-induction, cellular debris, soluble protein, wash, and eluate stages were run out on 8% SDS-PAGE gels and subsequently stained with Coomassie blue to confirm the presence of the purified fusion protein in the eluate.

Western Analysis

Total skeletal muscle was collected from 12-week old WT, Flox, and CASK SkM cKO mice and homogenized in Newcastle buffer (75 mM Tris, pH 6.8, 3.8% sodium dodecyl sulfate (SDS), 4 M urea, 20% glycerol). Protein concentrations were determined using the DC protein

assay (Biorad) followed by subsequent absorbance measurements at 750 nm wavelength. 50 µg of protein per sample was then run out on 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels at 80 V for approximately 3 hours. After separation, proteins were electrically transferred from SDS-PAGE gels to nitrocellulose (Whatman International) or polyvinylidene fluoride (PVDF, Bio-Rad) at 80 V for 90 min using a wet transfer apparatus (Bio-Rad).

Nitrocellulose western blots were then blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline plus 0.1% Tween-20 (TBST) and 1% normal goat serum for 1 hour. Following blocking, blots were then incubated in a primary antibody solution consisting of a primary antibody and 1% normal goat serum in TBST for 2 hours. Primary antibodies include CASK polyclonal (Zymed, 1:250), Dlg monoclonal (Transduction Laboratories, 1:250), polyclonal phosphoserine (Invitrogen, 1:250), polyclonal phosphotyrosine (Cell Signaling, 1:250), and polyclonal phosphothreonine (Millipore, 1:250). Blots were then washed 3 x 15 min in TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Jackson Laboratories, 1:10,000) in TBST with 1% normal goat serum for 1 hour. Chemiluminescence (Amersham) was used for detection of bound primary antibody on nitrocellulose.

PVDF blots were used for dual labeling with primary antibodies, and were first blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline plus 0.1% Tween-20 (TBST) and 1% normal goat serum for 30 min. Blots were then incubated overnight in a primary antibody solution consisting of two distinct primary antibodies raised in different species for dual labeling and 1% normal goat serum in TBST. Primary antibodies used include Dlg monoclonal (UC Davis, 1:250), polyclonal phosphoserine, and GluR2 monoclonal (UC Davis, 1:250). Blots were then washed 3 x 15 min in TBST and incubated with a fluorochrome-conjugated goat anti-rabbit

secondary antibody (Licor Biosciences, 1:10,000, detectable at a wavelength of 680 nm) and a fluorochrome-conjugated goat anti-mouse secondary antibody (Licor Biosciences, 1:10,000, detectable at a wavelength of 800 nm) in TBST with 1% normal goat serum for 1 hour. Bound primary antibody was detected using the Odyssey[®] Infrared Imaging System (Licor Biosciences).

Proteomic Analysis

300 µg of homogenized total skeletal muscle protein (quadriceps and hindlimbs) from each genotype was passively rehydrated onto 11 cm, pH 4-7, isoelectric focusing (IEF) strips (BioRad) and then subjected to first dimension isoelectric focusing using a PROTEAN IEF machine (BioRad). The isoelectric focusing conditions were as follows: start voltage at 0 V, end voltage was 8,000 V, and the IEF was run for 20- 35,000 V-hr. Protein on the strips then was run in the second dimension on a 8-16% gradient gel (BioRad) for 65 min at 200 V, then transferred and probed with phosphoserine as described previously in the western analysis section. Spot differences were then observed between the different genotypes. Proteomic analysis was conducted by Jamie Sanford

Results

F.L. Dlg Cannot be Effectively Isolated via a GST-Fusion Protein Protocol

To begin the characterization of the role of the CaMK domain in skeletal muscle, we began work to perform kinase assays to determine the capacity of the CaMK domain of CASK to phosphorylate Dlg *in vitro*. To perform kinase assays *in vitro*, vectors containing GST-CASK or GST-Dlg were cloned into *E. coli*, and protein synthesis was induced in culture via IPTG induction of the *lac operon*. The fusion proteins were eluted through glutathione beads and the

protein isolates were run out on an 8% SDS-PAGE gel and stained with Coomassie blue. Results consistently revealed successful isolation of the full length CASK fusion protein in the eluate fraction. However, the Dlg fusion protein was fragmented during isolation and Coomassie staining revealed that the full length protein could not be retrieved. Western analysis on the different fractions retrieved during protein isolation with a polyclonal Dlg antibody was also unable to detect the isolated full length protein in the eluate fraction (data not shown).

A Loss of Non-Synaptic CASK Affects the Phosphorylation Levels of Other CASK Interactors

Because generation of full-length fusion proteins was unsuccessful, we next focused on more global approaches to determine what proteins CASK phosphorylates in muscle using our transgenic and knockout mouse models. In order to determine whether loss of CASK in cytoplasm results in global phosphorylation differences, we performed western analysis on a wide array of CASK protein interactors on skeletal muscle homogenates and microsomes from both the HSA-Cre CASK SkM cKO mice and their WT/+ littermates. We first probed nitrocellulose blots with one of either phosphoserine (Invitrogen, 1:250), phosphotyrosine (Cell Signaling Technology, 1:250), or phosphothreonine (Millipore, 1:250). Staining with phosphoserine and phosphothreonine revealed distinct differences in the levels of phosphorylation among various proteins between total skeletal homogenates of WT/+ and HSA-Cre CASK SkM cKO mice (figure 4-1). Staining with phosphotyrosine displayed a similar number of differences in phosphorylation levels (data not shown). These results suggest that the phosphorylation levels of other protein interactors are altered due to a loss of cytosolic CASK in skeletal muscle.

Next, we performed western analysis on the same samples and transferred protein gels to PVDF membranes. PVDF blots were then dual-labeled with phosphoserine and Dlg or labeled with the glutamate receptor subunit GluR2. Dual labeling with phosphoserine and Dlg revealed that Dlg is indeed phosphorylated in skeletal muscle at one of its serine residues (figure 4-2). Staining for GluR2 revealed that the glutamate receptor subunit is heavier in CASK FL+ homogenates than in CASK Δ^+ homogenates, suggesting that GluR2 may be phosphorylated in response to an overexpression of the FL-CASK protein (figure 4-3).

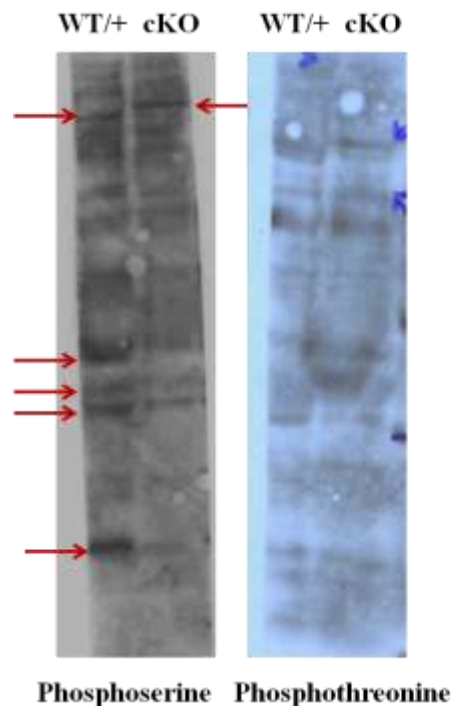


Figure 4-1: Western analysis on WT/+ and HSA-Cre CASK SkM cKO skeletal muscle homogenates with phosphoserine and phosphotyrosine reveals differences in phosphorylation of certain proteins between genotypes. Arrows point to significant varying levels of phosphorylation between genotypes.

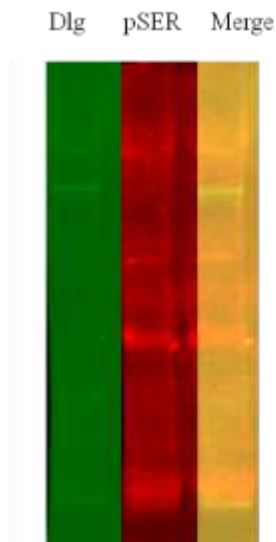


Figure 4-2: Western analysis of WT/+ microsomes dual-labeled for Dlg and phosphoserine. Analysis shows that Dlg is indeed phosphorylated *in vivo* in skeletal muscle at a serine residue.



Figure 4-3: Western analysis of WT/+ homogenates stained for the glutamate receptor subunit GluR2. Staining revealed a lower band in the CASK Δ^+ lane when compared to the CASK FL+ lane, suggesting that GluR2 may be phosphorylated in response to an overexpression of the FL-CASK protein.

Levels of Other Phosphorylated Proteins are Affected in Response to Levels of CASK Expression

To determine if the altered levels of protein phosphorylation was due to a loss of non-synaptic CASK, proteomic analysis via 2-dimensional gels was run for CASK FL+, CASK Δ +, and transgenic negative (Tg-) skeletal muscle homogenates (figure 4-4). Proteomic analysis separates proteins based on both size and charge so that individual protein spots can be visualized. Analysis revealed drastic spot differences between the three genotypes, suggesting that CASK expression plays a major impact on the expression and post-translational modifications of other proteins in skeletal muscle.

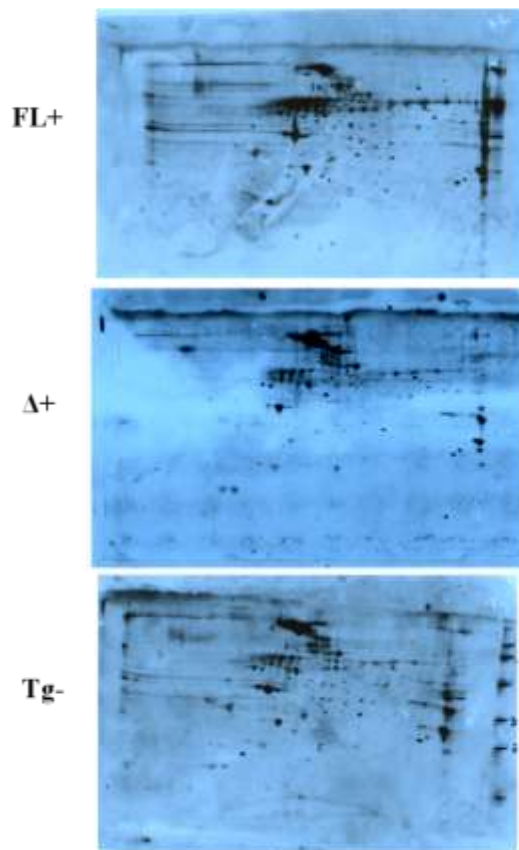


Figure 4-4: Proteomic analysis of FL+, Δ +, and Tg- skeletal muscle homogenates. 2-Dimensional gels were run for each genotype and spot differences were observed. The varying degrees of spot size and location, notably in the FL+ homogenate, illustrate that other protein levels and post-translational modifications are influenced by CASK expression. Figure adapted from Sanford *et. al.* 2005.

CASK May Be Phosphorylated and Interacts with Cdk5 in Skeletal Muscle

To begin to determine whether CASK is phosphorylated in skeletal muscle, we performed western analysis on WT subcellular fractions of nuclear extract (NE) and cellular extract (CE) and probed with CASK polyclonal antibody (figure 4-5). Results showed that the CASK band was lower in the NE fraction, implying that the CASK protein is heavier in the cytosol than in the nucleus. We hypothesize that the difference in weight of the CASK protein between the two fractions implies that CASK is predominantly phosphorylated in the cytosol while CASK in the nucleus remains unphosphorylated.

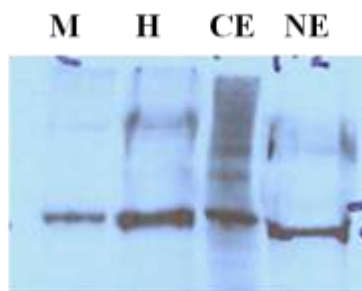


Figure 4-5: Western analysis of CASK expression in transgenic negative (Tg-) skeletal muscle homogenates and subcellular fractions. Microsomes (M), total skeletal muscle homogenates (H), cytosolic extract (CE), and the nuclear extract (NE) from Tg- mice were probed with anti-CASK. Results reveal that CASK is lower in the NE fraction, suggesting that CASK is phosphorylated in the cytosol and not in the nucleus.

Because CASK has been shown to be phosphorylated by Cdk5 in neuronal cultures, we began to study whether this was the case in skeletal muscle. To determine the degree to which CASK becomes phosphorylated in skeletal muscle, we first used western analysis to determine if Cdk5 expression was altered in response to changes in the expression levels of CASK (figure 4-6a). Western analysis was conducted on skeletal muscle homogenates from mice that

overexpressed the full length CASK transgene (FL+), mice that overexpressed a truncated portion of the CASK transgene (Δ +), wild type mice (Tg-), WT/+ mice, and HSA-Cre CASK SkM cKO mice. Results showed that Cdk5 levels remained relatively consistent across genotypes, suggesting that upregulating CASK does not result in a concomitant upregulation of Cdk5 in skeletal muscle.

To determine whether or not Cdk5 interacts with and therefore potentially phosphorylates CASK in skeletal muscle, immunoprecipitations were conducted using total skeletal muscle homogenates to illustrate the possible binding between CASK and Cdk5 *in vivo*. Immunoprecipitations were conducted with a CASK monoclonal antibody and bound and unbound fractions were run out on an SDS-PAGE gel, transferred to nitrocellulose, and probed for Cdk5 (figure 4-6b). Results revealed bands that correspond to the Cdk5 protein in the bound lane, suggesting that CASK interacts and binds with Cdk5 in skeletal muscle. Further analysis will be needed to determine whether this interaction translates to a phosphorylation of CASK by Cdk5 in skeletal muscle.

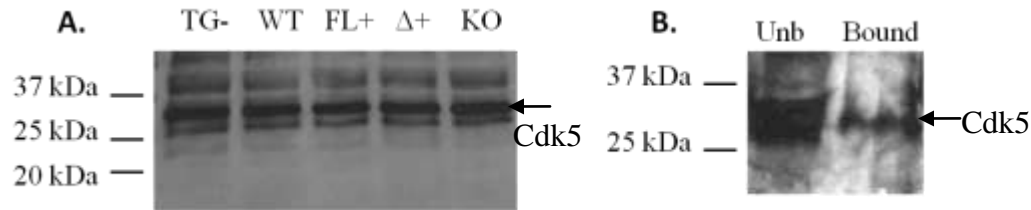


Figure 4-6: Western analysis illustrating the presence and interaction of Cdk5 with CASK in skeletal muscle homogenates. A.) Homogenates from transgenic negative mice (Tg-), WT mice, mice overexpressing the full length CASK transgene (FL+), mice overexpressing a truncated portion of the CASK transgene (Δ +), and HSA-Cre CASK SkM cKO (KO) mice were run out and probed with Cdk5 antibody. Results show that Cdk5 levels remain relatively unaltered across all genotypes. B.) CASK immunoprecipitations probed with Cdk5 illustrate that CASK binds and interacts with Cdk5 in skeletal muscle.

Discussion

CASK is a unique protein, in that it contains all of the standard binding domains characteristic of MAGUK proteins, as well as a specialized CaMK domain capable of protein phosphorylation (Hsueh *et. al.* 2000, Nix *et. al.* 2000). It has been postulated that this arrangement of domains gives CASK an adaptive function in skeletal muscle development. First, CASK contains a PDZ domain that may bind to protein substrates and hold them in place for phosphotransfer by the CaMK domain (Mukherjee *et. al.* 2008). The SH3 and GK domains of CASK are capable of intramolecular interactions, which may allow CASK to adopt a number of different catalytically active conformations to adapt to the different environments of separate tissues or throughout development (MacLennan and Edwards 1990, McGee and Bredt 1999, McGee *et. al.* 2001, Nix *et. al.* 2000, Shin *et. al.* 2000, Tavares *et. al.* 2001). In addition, the CaMK domain has been shown to be catalytically active without a functional Mg^{2+} binding site, a cofactor typically essential for kinase activity. This may allow CASK to be a predominant

kinase in developing skeletal muscle when Mg^{2+} levels are particularly low (Mukherjee *et. al.* 2008).

Our findings that CASK interacts with Cdk5 in skeletal muscle, coupled with the evidence that phosphorylated CASK is implicated in synaptogenesis, may suggest a crucial role for CASK phosphorylation in neuromuscular junction formation and/or stabilization. Cdk5 has been shown to catalyze phosphotransfer to a number of proteins involved in cell signaling, development, and other biochemical pathways, and its involvement with CASK could implicate an important role CASK has in cell signaling and NMJ development (Samuels *et. al.* 2007). Further evidence and the study of the interaction between Cdk5 and CASK, however, would be necessary to determine a correlation between CASK phosphorylation and NMJ scaffolding and formation *in vivo*. Experiments may include the cotransfection of CASK and Cdk5 in C2C12 mouse myoblast cells followed by western analysis to see if CASK phosphorylation takes place, or *in vitro* kinase assays between Cdk5 and CASK to confirm the ability for Cdk5 to phosphorylate CASK.

Our western analysis between nuclear extract and cytosolic extracts with the CASK antibody helps to show that CASK itself is indeed phosphorylated in skeletal muscle. However, further experimentation is necessary to confirm these data. Additional attempts to perform western analysis on homogenates and stain with a functional phospho-CASK specific antibody would help to prove that CASK is indeed phosphorylated in skeletal muscle, and that this phosphorylation varies depending on the subcellular localization of CASK. Proteomic analysis on homogenates with CASK followed by mass spectrometry will also help to determine any post-translational modifications or phosphorylation that CASK may be subjected to.

We also looked at the degree to which different levels of CASK expression affects the phosphorylation of other proteins in skeletal muscle. Using four models that represent different levels of CASK expression (FL+, Δ +, cKO, and Tg-), we were able to observe differences in protein levels and modifications in each. Western analysis with a phosphoserine antibody revealed differences in protein phosphorylation between WT and cKO mice. A more in-depth proteomic analysis revealed significant spot differences between FL+ and Tg- homogenates, and to a lesser extent, differences between Δ + and Tg- homogenates. Further analysis would involve cutting individual spots out of the gels and submitting them to the Proteomic Core Facility for mass spectrometry. This would allow us to determine the identity of the individual spots so we could determine which proteins were being altered due to the overexpression of the full-length CASK protein.

In addition to mass spectrometry, further analysis of the capacity of the CaMK domain and its role in phosphorylation in skeletal muscle would better help us to characterize the role that CASK plays in catalytic phosphotransfer *in vivo*. While we were unable to successfully isolate full-length Dlg fusion protein, full-length CASK was successfully isolated, and kinase assays could be performed on a number of other known CASK protein interactors in skeletal muscle. These interactors include subtypes of the inward-rectifying potassium channel family, subunits of the glutamate receptors, other MAGUK proteins, and NMDA receptor subunits. Kinase assays on any of these proteins would help to illustrate the role that the CaMK domain plays in cell signaling and phosphorylation.

While we have made progress in determining the role of CASK phosphorylation and its kinase activity in skeletal muscle, there is still much work to be done. In addition to experiments involving further proteomic analysis and kinase assays, we can work to determine how a

phosphorylation in CASK affects its function. Perhaps CASK phosphorylation plays a role in how it functions in receptor trafficking or where it localizes. For example, we can mutate phosphorylated residues of CASK to see if it still localizes to the NMJ. While we have only skimmed the surface on CASK's phosphorylative abilities in skeletal muscle, this work lays the foundation for determining the differences between the roles of CASK and phosphorylated CASK in skeletal muscle.

V

Discussion and Future Directions

The Adaptive Role of CASK in Skeletal Muscle

The studies conducted in this thesis set out to define the role that the neuronal scaffolding MAGUK protein CASK plays in mammalian skeletal muscle. To this end, we used the Cre/lox recombination system to develop two distinct conditional knockout models in which non-synaptic CASK was ablated in skeletal muscle (HSA-Cre CASK SkM cKO) or CASK was completely knocked out in skeletal muscle (Myf5-Cre CASK SkM cKO) (Hoess *et. al.* 1985, Sternberg *et. al.* 1981). This differentiation was possible due to the fact that the HSA promoter is most likely not expressed in synaptic nuclei, while the Myf5 promoter is transcribed before synaptic development and throughout premature skeletal muscle fibers.

A characterization of the HSA-Cre CASK SkM cKO mouse model led us to conclude that there was no obvious degenerative phenotype present when compared to their WT/+ littermate controls. However, we did find alterations in the levels, localizations, and post-translational modifications of other CASK interactor proteins in skeletal muscle homogenates that may be attributed to a loss of non-synaptic CASK. To further determine which proteins are in fact phosphorylated or up-/down-regulated, a more in-depth proteomic analysis is required, including, but not limited to, 2-dimensional gel analysis in which spot variations between genotypes can be cut out and identified via mass spectrometry.

While this model did not exhibit a pathological phenotype, the Myf5-Cre CASK SkM cKO model, in which CASK is completely knocked out from skeletal muscle, may show a degenerative histology. Initial analysis of this model shows that CASK ablation results in underdeveloped skeletal muscle fibers in prenatal (e16.5) mice when compared to WT/+

littermate controls. These results have been confirmed by both histological staining with hemotoxylin and eosin dyes and immunohistochemical staining with the dystrophin antibody and the fluorescent nuclear dye DAPI. Prenatal Myf5-Cre CASK SkM cKO mice may show a gross phenotype illustrated by their relatively smaller size when compared to littermate controls of the same age. Additionally, there were fewer Myf5-Cre CASK SkM cKO mice observed than predicted by Mendelian genetics when compared to all other littermates. Both of these observations suggest that the Myf5-Cre CASK SkM cKO mice may be dying *in utero*. However, a much more in-depth phenotypic characterization is required to confirm these results. This may require functional analysis to determine the strength of the developed skeletal muscles in the prenatal Myf5-Cre CASK SkM cKO mice.

A dynamic contrast exists between the phenotypes of the HSA-Cre cKO model and the Myf5-Cre cKO model. While a loss of non-synaptic CASK results in no skeletal muscle pathology, a loss of synaptic CASK may result in impaired skeletal muscle development and prenatal lethality. A major role CASK has been shown to play in neuronal cultures is to function in synaptic scaffolding, receptor clustering to the neuronal synapses, and phosphorylation (Hata *et. al.* 1996, Leonoudakis *et. al.* 2004, Mukherjee *et. al.* 2008, Sanford *et. al.* 2004). We hypothesize that the role of CASK at the NMJ is most likely similar to the role that CASK plays at the synaptic cleft in neurons, and that it functions in the formation and maintenance of the NMJ. Further experiments to test this hypothesis would include a comparison of the two models to differentiate the cytoplasmic functions of CASK from the synaptic functions of CASK. Physiology experiments can be performed to determine if synaptic transmission is altered between the two models. We can also do studies with primary tissue culture from the two models

to determine where CASK interacts with protein interactors such as glutamate receptor subunits and potassium channel subtypes.

Further analysis of the Myf5-Cre CASK SkM cKO model is necessary to definitely determine why a total loss of CASK is responsible for a degenerative phenotype while a loss of non-synaptic CASK is not. Perhaps the amount of CASK that remains expressed at the NMJ in the HSA model is sufficient to develop functional skeletal muscle. CASK may play an important role in NMJ formation during myogenesis through the trafficking of receptors and channels to the NMJ or the phosphorylation of other MAGUKs and synaptic scaffolding proteins in the Mg^{2+} -poor environment where other kinases are slower to function (Hata *et. al.* 1996, Leonoudakis *et. al.* 2004, Mukherjee *et. al.* 2008, Sanford *et. al.* 2004). Immunohistochemical analysis of the glutamate receptor subunits, the potassium channels, and other known CASK protein interactors in cell culture will be necessary to determine whether these essential components of the NMJ are being localized normally to the junction without the trafficking capabilities of CASK. However, at the present time, we can only infer from the data that synaptic CASK alone is sufficient for normal muscle development, and a loss of CASK at the NMJ results in abnormal fiber organization and differentiation.

CASK and Protein Phosphorylation

Another aim of this study was to determine the phosphorylative capacity of CASK in skeletal muscle, and what function phosphorylation of CASK itself plays in skeletal muscle function and development. In neuronal cultures, it has been shown that the CaMK domain of CASK is capable of phosphorylating the synaptic scaffolding protein neuroligin-1, a pre-synaptic protein imperative in the scaffolding of the neuronal synapse (Mukherjee *et. al.* 2008). A focus

of this aim was to determine whether CASK played a similar phosphorylative role in skeletal muscle and the development of the NMJ. The MAGUK protein Dlg, a known CASK interactor in the central nervous system and skeletal muscle was a good initial candidate for phosphorylation by CASK in skeletal muscle (Massimi *et. al.* 2006, Gardner *et. al.* 2006, Zhang *et. al.* 2007). While full-length Dlg fusion protein was not successfully isolated, studies on the role that altered CASK protein expression plays on the phosphorylation of Dlg and other candidate protein interactors was studied via western and 2-dimensional proteomic analyses. We found that the phosphorylation of a number of proteins is altered in the CASK FL+ and CASK Δ^+ models as compared to the wild type Tg- models. We also showed that Dlg is indeed phosphorylated at one or more serine residues in skeletal muscle. However, further proteomic analyses must be completed to determine what other specific proteins are altered and how in response to CASK overexpression.

In addition to the ability of CASK to serve as a functional kinase, CASK has been shown to be phosphorylated by the protein kinase Cdk5 in neuronal cultures (Samuels *et. al.* 2008). Our lab showed through immunoprecipitation that CASK and the protein kinase Cdk5 interact in skeletal muscle, and we hypothesize that Cdk5 plays a similar role in CASK phosphorylation in skeletal muscle tissue. This hypothesis is supported by our observation via western analysis that CASK is heavier in a cellular extract fraction of total skeletal muscle homogenate than in a nuclear extract fraction. This suggests that CASK is phosphorylated in the cytosol, but remains unphosphorylated in the nucleus. CASK phosphorylation in the cytoplasm may be significant as a means of cell signaling or adopting a conformation conducive to the binding and trafficking of protein channels receptors to the NMJ.

Limitations

There are several limitations within the findings presented in this thesis. First and foremost, the HSA promoter did not knock out CASK completely in skeletal muscle, which was our initial hope. However, we have generated the Myf5-CASK cKO mice to rectify this. Another major setback is the fact that the Myf5-CASK cKO mice die *in utero*, making it very difficult to study the role of CASK at the mature NMJ due to timing issues, as the mature NMJ isn't fully formed until 2 weeks following birth. Experiments can be performed in primary cell culture, but this may prove difficult as well since CASK is not recruited to the NMJ by normal agrin induction of acetylcholine receptor clustering. However, recent methods have been published in which co-culture with motor neurons is possible to study CASK's function at the NMJ (Das et. al. 2010, Guo et. al. 2010). Another limitation was our inability to isolate full-length Dlg fusion protein to perform kinase assays. However, future experiments can be conducted in cell culture to determine the phosphorylative capacity of the CaMK domain of CASK. In addition, our CASK antibodies have been unreliable and reveal a high degree of background staining. However, we have yet to try CASK polyclonal antibodies from Santa Cruz Biotechnology, LifeSpan biosciences, and Origene, which may solve this dilemma.

Conclusion

The data provided in this thesis supports the hypothesis that CASK plays a dynamic role in normal skeletal muscle function and development, and that CASK is essential for the normal differentiation of skeletal muscle fibers. We have shown that a loss of cytoplasmic CASK results in no aberrant muscle pathology, but alters the levels and localizations of other CASK protein interactors in skeletal muscle, the consequences of which have yet to be determined. We have

also demonstrated that a complete ablation of CASK in skeletal muscle may be responsible for a degenerative muscle pathology and abnormal muscle differentiation and development. This data, together with the evidence that CASK participates in protein phosphorylation, suggests an adaptive role for CASK in developing skeletal muscle and at the NMJ. Further delineation of the functions of CASK in developing skeletal muscle may help to elucidate the mechanisms behind developing neuromuscular diseases.

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